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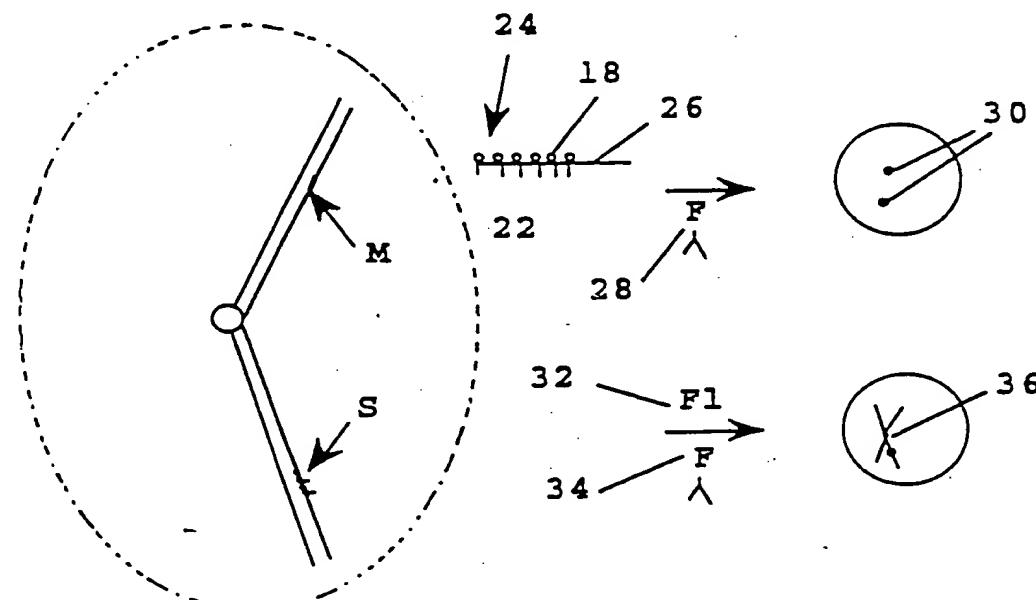
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(54) Title: IN SITU HYBRIDIZATION METHOD



(57) Abstract

A method of identifying the presence of a known target sequence in nucleic acid contained in a fixed cellular or subcellular biological structure. By adding a stable, reporter-labeled RecA/single-stranded probe complex to the cellular or subcellular structure, the target sequence can be effectively labeled by *in situ* hybridization, allowing the target sequence to be visualized histologically and microscopically or detected by *in situ* cytometry or cell sorting flow techniques.

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## DESCRIPTION

IN SITU HYBRIDIZATION METHOD

5 This application is a continuation-in-part of co-owned, co-pending U.S. Application 07/755,291, filed 4 September 1991.

10 1. Field of the Invention

The present invention relates to a diagnostic method for performing *in situ* hybridization with double-stranded DNA targets.

15 2. References

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### 3. Background of the Invention

In situ hybridization employs direct hybridization of a DNA probe with DNA or RNA in biological structures, typically permeabilized cells, subcellular fractions, or fixed chromosome preparations. Because the method can yield morphological information about the localization of specific-sequence target nucleic acid(s) in fixed biological structures, it is applicable to many areas of biomedical research, such as developmental biology, cell biology, genetics and particularly gene mapping, pathology and gene diagnostics.

In most applications, in situ hybridization is directed toward a target sequence in a double-stranded duplex nucleic acid, typically a DNA duplex associated with a pathogen or with a selected sequence in viral or cell chromosomal DNA. In this method, as it has been practiced heretofore, a single-stranded labeled probe is added to the permeabilized structure, which has been heated to a temperature sufficient to denature the target

duplex nucleic acid, and the probe and denatured nucleic acid are allowed to react under suitable hybridization, or reannealing conditions. After removal of unbound (non-hybridized) probe, the structure is processed for 5 examination for the presence of reporter label, allowing the site(s) of probe binding to target duplex nucleic acid to be localized in the biological structure, i.e., in the context of cell or subcellular morphology.

The method has been widely applied to chromosomal 10 DNA, for mapping the location of specific gene sequences, and distances between known gene sequences (Lichter, Meyne, Shan), for studying chromosomal distribution of satellite or repeated DNA (Weier, Narayanswami, Meyne, Moyzis, Joseph, Alexandrov), for examining nuclear 15 organization (Lawrence, Disteche, Trask), for analyzing chromosomal aberrations (Lucas), for localizing DNA damage in single cells or tissue (Baan) and for determining chromosome content by flow cytometric analysis (Trask). Several studies have reported on the 20 localization of viral sequences integrated into host-cell chromosomes (e.g., Harders, Lawrence, Lichter, Korba, Simon). The method has also been used to study the position of chromosomes, by three-dimensional reconstruction of sectioned nuclei (van Dekken), and by 25 double *in situ* hybridization with mercurated and biotinylated probes, using digital image analysis to study interphase chromosome topography (Emmerich).

Another general application of the *in situ* hybridization method is for detecting the presence of 30 virus in host cells, as a diagnostic tool (Unger, Haase, Noonan, Niedobitek, Blum). In certain cases where the number of virus particles in the infected cell is very low, it may be necessary to first amplify viral sequences by *in situ* adopted polymerase chain reaction (PCR) 35 methods (Haase, 1990, Buchbinder).

The *in situ* hybridization method described above has a number of limitations. The most serious limitation is the requirement for denaturing the duplex target DNA, to form the necessary single-stranded form of the target.

5 Denaturation typically is performed by heating the sample or treating with chemicals and heat. The heat treatment can produce spurious and unwanted changes in the nucleic acid being examined, related to structural changes and nucleic acid reassociation with repeated sequences within

10 the DNA. The repeated DNA sequences can randomly reassociate with one another. The step also adds to the time and effort required in the method.

Secondly, where the target sequence of interest is present in very low copy number, the method is limited, 15 by renaturation kinetics, to long renaturation times. Even then, the method may be incapable of producing probe/target renaturation events at low target concentration. This limitation may be partly overcome, as indicated above, by first amplifying the target duplex 20 in *situ* by modified PCR methods. However, the PCR approach involves additional steps, and may be unsuitable for many *in situ* studies, such as those involving localization of genomic chromosomal DNA sequences.

25 4. Summary of the Invention

It is therefore a general object of the present invention to provide an *in situ* hybridization method, for use in detecting and/or localizing target nucleic acid, typically duplex DNA, in a fixed biological structure, 30 which (a) does not require heat denaturation of the target duplex, and (b) is not limited in target duplex copy number by renaturation kinetics.

The present invention includes a method of identifying the presence of a known target sequence in a 35 double-stranded nucleic acid contained in a cellular or

subcellular biological structure, in a specific morphological relationship with the structure. The method includes adding to the structure, a probe complex composed of RecA protein stably bound to a single-stranded, reporter-labeled nucleic acid probe which is complementary to one of the strands of the duplex target sequence, under conditions in which the complex can contact the duplex nucleic acid. The complex is allowed to bind to the target sequence under non-denaturing conditions. After removing unbound complex, the structure is examined for the presence of the reporter-labeled probe bound to the nucleic acid.

The complex is preferably stabilized by preparation in the presence of ATP $\gamma$ S. The probe may be labeled with a detectable reporter, such as a radiolabel, enzyme or fluorescence tag, or with a ligand, such as biotin or digoxigenin, which can be subsequently reacted with a reporter molecule specific for the ligand, and carrying a detectable reporter. The complex may also be stabilized using other cofactors including, but not limited to, ATP $\gamma$ S, GTP $\gamma$ S, ATP, dATP and a combination of ATP $\gamma$ S and ADP.

In one general application, the method is used for detection and localization of genomic sequence(s) in fixed chromosome DNA structure(s) in metaphase spreads. In one embodiment, the microscopic ultrastructure of the chromosomes is determined, for example, by fluorescence microscopy, using fluorescence banding patterns. The location of the bound complex in relation to the known ultrastructure is then determined independently, for example, by a fluorescence-labeled probe complex whose fluorescence excitation wavelength is different from that of the chromosome banding fluorescence. Alternatively, fixed cells or cellular structures are probed in suspension followed by flow cytometric or microscopic

analysis.

In another general application, the method can be used for detecting the presence of virus or integrated virus-specific genomic sequences in a host cell. The 5 binding of a fluorescence-labeled probe to the virus sequence may be determined by fluorescent microscopy or fluorescence activated cell sorting (FACS) or a light or fluorescent or laser scanning microscope. Where an enzyme label is used a light microscope can be used to 10 visualize colored (e.g., black) peroxidase or alkaline phosphatase product produced by the reporter enzyme.

Another embodiment of the present invention includes a method of identifying the presence of a known viral nucleic acid target sequence contained in a fixed 15 cellular or subcellular biological structure. Such known viral nucleic acid targets include known DNA viruses (such as hepatitis B virus) or RNA viruses that can have a detectable duplex nucleic acid phase in their life cycle. In this method, the fixed structures or 20 substructures can be incubated in 10 mM Tris-acetate buffer, pH7.5, at 55-60°C before the addition of the RecA probe complex in order to increase reaction efficiency: this step does not denature the cellular DNAs.

The present invention also includes a method of 25 detecting a single copy nucleic acid sequence, typically a duplex DNA sequence, contained in a cellular or subcellular biological structure. In this method the cellular or subcellular biological structure(s) are fixed. A probe complex (composed of RecA protein stably 30 bound to a single-stranded, reporter-labeled probe which is complementary the single-copy nucleic acid target sequence) is added to the cellular structure or substructure under conditions in which the complex can contact the nucleic acid target sequence. The complex is

then allowed to bind to the target sequence under non-denaturing conditions. Unbound complex is then removed from the structure and the structure is examined for the presence of the reporter-labeled probe bound to the 5 nucleic acid.

In this method of single-copy nucleic acid detection, the cellular structures or substructures can be fixed and analyzed in solution or on slides. The fixing can also include incubation of the fixed 10 structures or substructures in 10 mM Tris-acetate buffer, pH7.5, at 55-60°C. In the method of the present invention, the complex can be bound to the target sequence under non-denaturing conditions in reactions carried out for less than 2 hours.

15 The method of the present invention can also include the addition of accessory proteins, such as single-strand binding protein (SSB), topoisomerase I or topoisomerase II.

20 The present invention also includes kits containing components useful to carrying out the methods described above. One example for a kit for *in situ* detection of a known viral nucleic acid in a sample may include (i) a probe derived from the viral DNA sequences, (ii) RecA protein effective for coating the probe, and (iii) means 25 of detecting the binding of the probe to the known viral DNA in a sample. Such kits may also include RecA-protein coated DNA.

30 These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

##### 5. Brief Description of the Figures

Figures 1A and 1B are fluorescence photomicrographs

1 chromosome X alpha satellite DNA probe used for detection of decondensed or partially decondensed alpha satellite chromosomal centromeric DNA target sequences in native, nondenatured (1A) and heat-denatured (1B) methanol-acetic acid fixed interphase HEp-2 cell nuclei;

5 Figures 2A and 2B are fluorescence photomicrographs of alpha satellite DNA probe to chromosome 7 used for detection of decondensed chromosomal centromeric DNA target sequences in native, nondenatured (2A) and heat-denatured (2B) fixed nuclei in interphase HEp-2 cells;

10 Figures 3A and 3B are photomicrographs taken under fluorescence microscopy (3A) and phase microscopy (3B), at the same focus, showing the distribution of chromosome X alpha satellite DNA in a dividing fixed HEp-2 cell nucleus.

15 Figures 4A-4D illustrate steps for gene localization on a chromosome, employing the method of the invention;

20 Figures 5-10 show various types of chromosomal aberrations (upper frames A), and the corresponding fluorescence pattern which would be seen with such aberrations (lower frames B); and

25 Figures 11A-11C illustrate the steps in detecting virus infection of cells, by fluorescence activated cell sorting, in accordance with the invention.

30 Figure 12 presents a photograph of a cell preparation showing hybridization signal from fixed HEp-2 metaphase chromosomes hybridized with RecA-coated, biotinylated, nick-translated probe to human chromosome 1 alpha-satellite centromeric sequences.

35 Figures 13A to 13F show RecA-mediated native fluorescence *in situ* hybridization detection of unique p53 chromosome 17 tumor suppressor gene sequences in ATCC HEp-2 and HCC "Alexander" cells in suspension.

40 Figures 14A to 14D show RecA-mediated native fluorescence *in situ* hybridization detection of unique

p53 gene sequences in ATCC HEp-2 cell nuclei on slides.

Figures 15A to 15E show RecA-mediated native fluorescence *in situ* hybridization detection of Hepatitis B Virus (HBV) nucleic acid sequences in ATCC HCC "Alexander" cells in suspension.

Figures 16A to 16C show specificity of HBV target detection using RecA-mediated native fluorescence *in situ* hybridization detection in human HCC cells tested by competition hybridization.

10

#### 6. Detailed Description of the Invention

##### I. In situ Hybridization Method

This section describes the basic methodology of *in situ* hybridization, in accordance with the invention, as applied to various biological structures containing a duplex DNA target with a repeated or unique specific basepair sequence.

##### A. Preparation of Biological Structures for DNA Detection

The method of the invention is designed for detecting, by complementary-basepair hybridization, a selected target sequence in a biological structure containing a duplex nucleic acid, usually a DNA/DNA duplex nucleic acid. The biological structure is any morphologically distinct structure, such as a cell, sperm, parasite, subcellular fraction or chromosomal preparation containing the target nucleic acid.

The target duplex in the structure is typically chromosomal DNA, or nucleic acid duplex material associated with a viral, parasitic or bacterial pathogen, such as virus particles composed of viral duplex genome encapsulated or released from being encapsulated in viral coat proteins. Methods of preparing fixed biological structures, such as cells, nuclei, and chromosomal

preparations generally fall within those used in conventional *in situ* hybridization by DNA duplex denaturation and reannealing.

Briefly, the cellular compartment and DNA structure 5 may be further fixed or permeabilized by treatment with an organic solvent and acid or cross-linking agent to fix the structural components in their natural morphological relationship. Common fixatives include acetic acid, salts, methanol, formalin, paraformaldehyde, and 10 glutaraldehyde. After fixation, tissue sample may be prepared for slide presentation by embedding in wax or by freezing, followed by sectioning into thin slices.

More generally, the biological material is treated 15 with one or more of a number of agents capable of deproteinizing and/or delipidizing the structures. Such methods can involve the use of proteases, lipases, acid, organic solvents including alcohols, detergents or heat denaturation or combinations of these treatments. A common treatment involves one or more washes with 20 methanol:acetic acid.

Other pretreatments may be useful in reducing 25 background, such as use of inhibitors of non-specific binding of nucleic acids. For example, prehybridization with non-specific carrier DNA (e.g. salmon sperm) or RNA (e.g. tRNA), may act to reduce non-specific probe binding to the fixed DNA-target structure.

Cellular structures of interest may be individual 30 cells, obtained for example from cell culture, cells present in a tissue section or body fluid. Typically, cellular structures from a tissue are sectioned 35 cryogenically, then treated on a slide, as above, to permeabilize the section, such as by treatment with methanol:acetic acid. Cellular structures may be studied to determine intracellular localization of genomic target sequence(s), or for detecting the presence and/or

1 calcification of an infective organism, such as virus, bacteria, or parasite in the cells.

Subcellular structures, such as nuclei and mitochondria, can be prepared by conventional fractionation methods, such as isopycnic centrifugation, to obtain subcellular material in enriched or substantially purified form. Thereafter, the enriched structure preparation may be permeabilized and deproteinized, as above, probed either in solution or 10 affixed to a slide, as by drying.

Alternatively, the cells may be pretreated with 75 mM KCl, followed by treatment with methanol:acetic acid, to remove cytoplasm. This fraction, after purification may be further treated for probe hybridization. This 15 method is illustrated in Examples 3-4 and 12-14 for the preparation of HEp-2 cell nuclei for *in situ* hybridization.

Briefly in these examples, HEp-2 cells were pelleted by low-speed centrifugation and the pellet was 20 resuspended in 75 mM KCl for between 5 and 15 min for a desired amount of nuclear swelling to occur, followed by addition of ice cold methanol:acetic acid and centrifugation. After general further addition of ice cold methanol:acetic acid and gentle agitation of the 25 cells after each addition followed by centrifugation, cytoplasm was degraded from the nuclei. The resulting isolated nuclei preparation was resuspended in methanol:acetic acid, placed in 10  $\mu$ l aliquots on microscope slides, dried, and the slides stored at -20°C 30 for later use. Alternatively, cells can be harvested using standard conditions, washed in 1X phosphate buffered saline (PBS) and fixed in 100% methanol or 70% ethanol then stored at -20°C: these cells can be used in solution hybridization detection reactions.

Another structure of general interest is a fixed chromosome preparation, typically derived from cells in metaphase (Pinkel, Cherif). The preparation may contain the entire set of genomic chromosomes from the cell, such as the preparation in Figures 1A and 1B, or individual, isolated chromosomes, such as can be obtained by published methods (Lebo, McCormick) or chromosome fragments. The chromosomes are generally treated with methanol:acetic acid, placed on a slide, then affixed to the slide with drying.

A variety of other subcellular structures, such as mitochondria, or pathogenic structures including parasites isolated from cell or blood samples, such as virion particles, may also be prepared according to standard methods, and fixed and permeabilized for *in situ* hybridization as above.

#### B. Target-Specific DNA Probe

The probe used in the method is a single-stranded nucleic acid, usually a DNA strand probe, or derived by denaturation of a duplex probe, which is complementary to one (or both) strand(s) of the target duplex nucleic acid. The probe sequence preferably contains at least 90-95% sequence homology with the target sequence, to insure sequence-specific hybridization of probe and target. The single-stranded probe is typically about 100-600 bases long, although a shorter or longer polynucleotide probe may also be employed.

The probe may be constructed or obtained by one of a number of standard methods. Many probes, such as various satellite DNA sequences are commercially available in single-stranded or double-stranded form. Other probes can be obtained either directly from viruses, plasmids and cosmids or other vectors carrying specific sequences, or, if desired, by restriction digest of the source or

the probe DNA, such as a vector, followed by electrophoretic isolation of specific restriction digestion fragments. Probes obtained in this manner are typically in double-stranded form, but may, if required, be 5 subcloned in single-stranded vectors, such as an M13 phage vector.

Alternatively, the probe may be prepared in single-stranded form by oligonucleotide synthesis methods, which may require, for larger probes, forming subfragments of 10 the probe, then piecing the subfragments together.

The probe is labeled with a reporter or ligand or moiety which allows detection of the targeted sequence *in situ*. For autoradiographic detection, the reporter is a radiolabel, such as  $^{32}\text{P}$ -labeled probe formed, for example 15 by nick translation or polymerase chain reaction in the presence of labeled nucleotides.

For fluorescence detection, the probe may be labeled with one of a selection of fluorescence groups, such as FITC, BODIPY, Texas Red, or Cascade Blue which is 20 excitable in a specific wavelength, such as 490, 540, and 361 nm. The groups are derivatized to 3' or 5' probe ends or by incorporation or reaction at internal positions, according to standard methods (Urdea, Keller, Zischler).

25 Alternatively, the probes may be labeled with a ligand-type reporter, such as biotin (Weier), digoxigenin (Zischler), or bromodeoxyuridine (BrdUrd) or other modified bases including fluorescein-11-dUTP (Boehringer-Mannheim) (Kitazawa). The probe reporter groups are 30 detected, *in situ*, by reaction of the hybridized probe with a secondary reporter molecule which (a) binds specifically and with high affinity to the probe ligands, and (b) contains a detectable reporter. The binding moiety of the secondary molecule may be avidin or

streptavidin, for binding to biotinylated nucleotides, anti-digoxigenin antibody, for binding to digoxigenin-labeled nucleotides, and anti-BrdUrd antibody for binding to BrdUrd-labeled probe.

5        The detectable reporter in the secondary molecule is typically a fluorescence label, but may also be a radiolabel, for autoradiographic detection, an antibody, an enzyme, for colorimetric or chemiluminescence detection in the presence of a suitable substrate, or  
10      colloidal gold (Narayanswami) for use in electron microscopic visualization.

C. RecA and mutant RecA803 protein purification:

15      RecA and RecA803 proteins, for use in forming the RecA/probe complex used in the invention, are preferably isolated from overproducing strains, such as *E. coli* strains JC12772 and JC15369 (obtained from A.J. Clark and M. Madiraju). These strains contain the RecA coding sequences on a "runaway" replicating plasmid vector  
20      present at high copy numbers per cell. The RecA803 protein is a high-activity mutant of wildtype RecA (Madiraju).

25      The RecA proteins can be purchased from Pharmacia or purified using fast protein liquid chromatography (FPLC) on a hydroxylapatite column followed by an anion (Mono Q) exchange column. The isolation procedure combines and modifies published procedures (Shibata et al., Griffith). Details are provided in Example 1.

30      The standard assays for monitoring the protein purification include assay of 38,000-dalton RecA protein by SDS-polyacrylamide gel electrophoresis (PAGE) (Pharmacia Phastgel system), enzyme assay of ssDNA-dependent ATPase activity using [ $\gamma$ -<sup>32</sup>P] ATP and PEI cellulose thin-layer chromatography developed in a

solvent of 0.5 M LiCl and 0.25 M formic acid, assay of DNase, assay of D-loop activity with 500-mer oligonucleotide probe.

Analysis of total protein from JC12772 and JC15369 5 cell extracts by SDS-PAGE (denaturing conditions) shows that the 38,000-dalton RecA protein is the major protein produced in these strains.

The SDS-PAGE profiles of the final Mono-Q-purified RecA and RecA803 proteins showed a single 38,000-dalton 10 band, free of other cellular polypeptides as detected by silver staining.

#### D. Preparation of RecA DNA Probe Complexes

The duplex nucleic acid in the biological structure 15 of interest is reacted with a probe complex composed of RecA protein stably bound to the single-stranded probe. The complex is preferably prepared in a stabilized form in the presence of ATP<sub>γ</sub>S.

RecA protein coating of probes is normally carried 20 out as detailed in Example 2. Briefly, the probe, whether double-stranded or single-stranded, is denatured by heating at 95-100°C for five minutes, then placed in an ice bath for 20 seconds to one minute followed by centrifugation at 0°C for approximately 20 sec, before 25 use. Denatured probes can be placed in a freezer at -20°C; preferably, however, they are immediately added to standard RecA coating reaction buffer containing ATP<sub>γ</sub>S, at room temperature, and to this is added the RecA protein.

RecA coating of probe is initiated by incubating 30 probe-RecA mixtures at 37°C for 10-15 min. RecA protein concentration tested during reaction with probe varies depending upon probe size and the amount of added probe, and preferably ranges between about 2 to 25 μM. When 35 single-stranded probes are RecA coated independently of

5 their homologous probe strands, the mM and  $\mu$ M concentrations of ATP $\gamma$ S and RecA, respectively, can be reduced to one-half those used with double-stranded probes (i.e. RecA and ATP $\gamma$ S concentration ratios are usually kept constant at a specific concentration of individual probe strand, depending on whether a single- or double-stranded probe is used).

10 E. Probe Hybridization to Permeabilized Biological Structures

15 According to an important feature of the invention, sequence-specific binding of the RecA/probe complex to the target duplex contained in a biological structure is achieved by adding the probe complex to the structure, under non-denaturing conditions, i.e., below the denaturation temperature of the duplex DNA, and allowing the complex to contact the target duplex, typically for 1-4 hours at 37°C, until homologous binding of the probe complex to the target DNA sequence has occurred.

20 After probe binding to the target DNA sequence, the target structure is washed to remove unbound probe complex. In the usual case, where the probe reporter is a ligand, such as biotin, the washed structure is contacted with a detectable reporter molecule, such as 25 fluorescence-labeled avidin (FITC-avidin), to bind a detectable reporter to the target-bound probe. The sample material is then further washed to remove unbound reporter molecule. A variety of wash procedures are suitable. The structure is visualized or otherwise 30 viewed or detected by microscopy, fluorescence activated cell sorting, autoradiography, or the like, as for example described below.

35 The hybridization condition described in Example 3, for use in fluorescence-reporter detection of a biotinylated probe, are exemplary. Briefly, between 10-

20  $\mu$ l probe complex is applied to a fixed preparation on a glass slide. Glass coverslips are placed over the hybridization areas and sealed, and the reactions are incubated in a moist container in a 37°C 7% CO<sub>2</sub> incubator for between 1-4 hours. Following incubation, the coverslip rubber cement seal is removed and the slides, with coverslips are washed several times to loosen and remove coverslips and remove unbound probe complex.

5 The slides are placed in preblock solution, followed by (a) immersion in or application of FITC (fluorescein isothiocyanate)-avidin, in preblock solution in the dark, then in several washes to remove unbound FITC-avidin. An antifade agent, with or without counterstain such as propidium iodide, may be used to 10 reduce photobleaching.

15 If necessary the probe signal may be amplified by reacting the material on the slide with biotinylated anti-avidin antibody, followed by several wash steps and addition of FITC-avidin, to enhance the amount of 20 fluorescent signal bound to the target duplex.

25 The target structure is then examined for the presence of the reporter-labeled probe bound to the target nucleic acid, e.g., by fluorescence microscopy or laser scanning microscopy.

30 Figure 1A shows FITC signal from *in situ* hybridization of a chromosome X alpha satellite DNA probe to prepared, isolated HEp-2 cell interphase nuclei fixed on glass slides, in accordance with the present invention and without amplification, following the protocol detailed in Example 3. Chromosome X is estimated to contain about 5,000 copies/cell of the alpha satellite sequences (ONCOR literature). The biotinylated probe was reacted and post-labeled with FITC-avidin, as described above.

For comparative purposes, denatured biotinylated Chr # som X alpha satellite probe from the same stock used in the Figure 1A method was combined with formamide and dextran sulfate under traditional protocols and was hybridized to HEp-2 cell nuclei using prior art thermal denaturation (and renaturation) steps, with the results shown in Figure 1B. The procedure required several more hours for total preparation and hybridization time than the Figure 1A method, involved signal amplification, and generally gave a lower level of fluorescent signal through the nuclei.

A second method, reported in Example 4, shows that the method gives high-probe target specificity in a low copy number target sequence, without probe signal amplification. In this method, a chromosome-7 alpha satellite DNA/RecA complex is hybridized with HEp-2 interphase nuclei, as above. Chromosome 7 contains about 10 copies of the alpha satellite sequence probe used (ONCOR probe D7Z2).

Figure 2A shows the target signal pattern after probe binding and FITC labeling, in accordance with the invention. As seen, the probe is localized in two distinct spots, presumably corresponding to the two chromosome 7's containing the alpha satellite sequence.

Figure 2B shows the *in situ* hybridization probe bound target pattern achieved with the same probe, after amplification following prior art methods described above. Probe localization appears to be less specific than in the method of the invention. Further, total preparation and probe hybridization times were many hours longer.

A third method, reported in Example 5, demonstrates the ability to localize a target sequence within a nuclear volume relative to other targeted DNA sequences and/or the nuclear membrane, using a confocal laser

scanning microscope (Zeiss LSM-10). In this method, 100% methanol fixed HEp-2 cells were probed in suspension with the RecA/chromosome-X alpha satellite DNA probe complex, and labeled with FITC-avidin, as in Figure 1A above.

5 Figure 3A shows the pattern of probe binding in a dividing nucleus. To localize the bound probe, the same field was viewed by phase contrast microscopy, without changing the focus of the lens (Figure 3B). By examining the two photomicrographs, the relative position of the 10 nuclear membrane and nuclear division plane can be seen with respect to the probe-labeled chromosomes.

The method of the present invention also facilitates the detection of specific DNA sequences in metaphase chromosomes using native RecA-mediated fluorescence in situ hybridization. RecA coated biotinylated probe specific for human chromosome 1 alpha-satellite centromeric sequences was reacted with fixed HEp-2 cells on slides using the native RecA-mediated fluorescence in situ hybridization (Example 6). Before RecA-coated probe mix addition, cells were incubated at 60°C with 10 mM Tris-acetate (pH 7.5). This incubation step, below the denaturation temperature of cellular nucleic acid targets, improves the efficiency of the fluorescence in situ hybridization reaction. In Example 6, using this 20 incubation step 73% of all cell nuclei showed fluorescence hybridization signals. FITC hybridization signals were visualized using a Zeiss LSM in 488 nm argon-ion laser-scanning mode. The FITC hybridization signal is superimposed on the phase image of the 25 chromosomes to identify its position (Figure 12). Note that the FITC probe signal is, as expected, located at the centromere.

30 RecA-mediated fluorescence in situ hybridization also facilitates the detection of unique gene sequences. 35 RecA-coated biotinylated probes specific for the p53 gene

(Onc r) were reacted with fixed cells in suspension using native fluorescence *in situ* hybridization reactions (Example 7). FITC probe signals were observed with a Zeiss LSM in 488 nm argon-ion laser-scanning mode.

5 Signals were apparent without any amplification of signal (i.e., extra signal amplification steps). The results of this analysis are presented in Figure 13: Figure 13A, 13C and 13E, FITC hybridization signals; Figure 13B, 13D, and 13F, phase images of cells in 13A, 13C and 13E, respectively; Figure 13A to 13D, HEp-2 cells; and Figure 13E and 13F, HCC "Alexander" cells. The FITC hybridization signals in Figure 13E are superimposed on the phase image of the cell in Figure 13F. Note that all hybridization signals are within cell nuclei and that

10 FITC signals are often seen as pairs indicative of newly replicated DNA. The cell nucleus in Figure 13D appears to be in the process of dividing. The results demonstrate the sensitivity of the method of the present invention for detecting unique sequences in solution hybridization reactions.

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In addition to detection of unique sequences in solution hybridization reactions, the method of the present invention is also effective for the detection of unique gene sequences using fixed cells on slides.

20 RecA-coated biotinylated p53 probe (Oncor) was reacted with fixed HCC cells on slides using a native fluorescence *in situ* hybridization reaction (Example 8). This reaction contained topoisomerase II and was not incubated in buffer before probe addition (Example 8).

25 FITC probe signals were observed with a Zeiss LSM in 488-nm laser-scanning mode. Hybridization signals were apparent without any amplification of signal (i.e., extra signal amplification steps). Sample results are presented in Figure 14. In Figure 14: 14A and 14C, FITC signals; 14B and 14D, phase images of cells seen in 14A

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and 14C, respectively. Note that all hybridization signals are within the nucleus and signals often appear as pairs. The position of the signal pairs in the nucleus shown, for example, in 14A and 14B suggests that 5 in this nucleus the signal may represent a stage after DNA replication. These results demonstrate the sensitivity of the method of the present invention for detecting unique sequences using fixed cells in hybridization reactions.

10 In addition to the ability of the method of the present invention to be used for the detection of unique cellular gene sequences, the method can also be used for the detection of unique viral nucleic acid sequences. RecA-coated HBV DNA probes pAM6 and "BIOPROBE" were 15 reacted with 100% methanol fixed cells in suspension using a native fluorescence *in situ* hybridization reaction (Example 9). Both probes used in these experiments detected HBV sequences in the human HCC cells with high efficiency ("BIOPROBE", 81%; pAM6, 95%). FITC 20 hybridization signals were observed with a Zeiss LSM in laser scanning mode. In Figure 15, the observed FITC signals from the HBV probes are shown superimposed on the phase images of the cells: 15A and 15B, "BIOPROBE"; 15C-15E, pAM6 probe. Note that all signals appear to lie 25 within the nuclear region. Both DNA probes generated multiple FITC hybridization signals in each HCC cell nucleus. The "BIOPROBE" signals appear less intense than the pAM6 probe signals. This is likely due to the size of the probes used. A RecA-facilitated pairing reaction between single-stranded probe(s) and linear duplex target DNAs in solution increases in efficiency 30 with increasing probe strand size: single-stranded "BIOPROBE" strands average <250bs and pAM6 single-strands average 300-500 bases in size. This difference might 35 also be due to the fact that the probes contain HBV

genomes of different serotypes ("BIOPROBE", adr-4; pAM6, adw). These results indicate that the method of the present invention is useful for the detection of viral DNA sequences. Probes specific for any viral DNA target of interest can be generated, RecA-protein coated, and used in the *in situ* hybridization method of the present invention. In addition to fluorescent detection a number of other detection methods might be used including, but not limited to, the following: chemiluminescence (Tropix Inc., Bedford MA) and radioactivity.

10. The method of the present invention also has a good specificity of target detection. The specificity of the present method was examined as follows. Thirty ng of RecA coated single-stranded biotinylated HBV probe (pAM6) was reacted with ATCC HCC "Alexander" cells using a standard native in suspension fluorescence *in situ* hybridization protocol (Example 10).

15. The specificity of the reaction signal for HBV targets was tested by adding 240 ng of either excess RecA-coated single-stranded non-biotinylated homologous DNA, or 240 ng of nonhomologous competitor DNAs (Example 10). Biotinylated HBV probe and non-biotinylated HBV and  $\phi$ X174 competitor DNAs were nick-translated under the same conditions to insure that they were of a similar size (average 400-500 bs). Unlabeled human placenta DNA, (100-120 bp fragments) was obtained from Oncor ("BLOCKIT DNA"). The results (Table 1; Example 10) show that only homologous HBV DNA, not heterologous DNAs, specifically competes with the biotinylated HBV DNA probe signal.

20. Representative cells from the competition experiments described in Table 1 are shown in Figure 16. In the figure: 16A, Biotinylated HBV probe + excess unlabeled HBV DNA; 16B, Biotinylated HBV probe + excess

unlabeled φX174 DNA; and 16C, Biotinylated HBV probe + excess unlabeled human placenta DNA. FITC probe signals were observed with a Zeiss LSM in laser scanning mode. The observed FITC signals from the HBV probes are shown 5 superimposed on the phase images of the cells. Note that it is clear from the signal and cell images that homologous HBV DNA specifically competes with the biotinylated HBV DNA probe signal but heterologous DNA does not compete. Thus, the RecA-facilitated native 10 fluorescence *in situ* hybridization reaction detects specific nucleic acid targets that are homologous to labeled probe DNA.

From the foregoing, it will be appreciated how various objects and features of the invention are met. 15 The invention provides a simplified and less time consuming procedure(s) for localizing target sequence in a biological structure. The method reduces artifacts by eliminating the need for a heat denaturation step and by reducing the need for signal enhancement, and allows more 20 rapid and well defined detection of target sequences, including target sequences of low copy number.

In particular, the method allows detection of low-copy sequences without the requirement to first amplify the sequences. A comparison of Figures 2A and 2B 25 demonstrate that this feature greatly enhances the specificity and resolution of the method over prior art approaches. Since most gene mapping and chromosomal studies are expected to involve specific low-copy sequences, the present method provides an important 30 advantage for diagnostic gene mapping studies, as well as for diagnostic application involving unique or low-copy numbers of various pathogen sequences. These later applications are described in Section II below.

Further, the methods described herein are 35 efficacious for the detection of (i) unique, i.e., single

copy, gene sequences, and (ii) unique or multiple viral nucleic acid sequences, in hybridization reactions carried out in solution and on slides.

As disclosed in the co-owned patent application for "Diagnostic Applications of Double D-Loop Formation" filed on even date herewith, stable RecA-coated probes prepared from duplex DNA fragments can form double-probe hybrid structures with target duplex DNA. Although such double-probe structures have not been shown for probe binding under *in situ* hybridization conditions, the presence of such structures, if formed, could be exploited to effectively double the amount of signal produced at the *in situ* target site. Further, the two probes could be labeled with different reporter groups, for example, fluorescent probes with different absorption or emission peaks, so that target sites containing both probes could be distinguished from sites containing one probe only.

## 20 II. Applications

One general application of the invention is for diagnostic use in locating and visualizing a selected gene or regulatory sequence in a chromosome, and/or in a particular region of the chromosome. The target gene or sequence may be one which (a) generates a selected gene product, (b) is suspected of performing a critical cell-control function, such as that of a ribosome, an oncogene, or a tumor suppressor gene, (c) is related to a repeat sequence, (d) is suspected of containing a genetic defect which prevents expression of an active gene product, (e) may be related in chromosome position to a marker probe region with a known map position, and/or (f) may represent an integrated or non-integrated viral sequence, such as a DNA-hepatitis virus (e.g., Hepatitis B Virus (HBV) (Ono, et al.; Fujiyama, et al.; Galibert,

et al.) in fixed chromatin or fixed virions.

The diagnostic probe used in the method may be obtained, in some cases, from available plasmids, cosmids, viruses or other vectors, such as from human genomic libraries or may be chemically synthesized. Where the gene product is available, the probe may be generated by sequencing enough of the protein product to generate probes for PCR amplification, and amplifying and tagging the corresponding gene sequence in genomic DNA using the probes in a PCR format. The amplified gene material can be purified by electrophoresis and used directly as the probe, or cloned into suitable vectors, using standard protocols.

In a typical method, the nuclei are derived from cells staged in metaphase, using well known methods, then fixed and "dropped" on a glass slide to produce a metaphase chromosomal spread. Alternatively, the chromosome material under investigation may be a spread of an isolated individual chromosome(s).

Figure 4A shows a single metaphase chromosome 10 which may be in isolated form or part of a field containing an entire set of somatic-cell chromosomes. The chromosome contains a known marker region 12 (gene site M) whose map location on the chromosome is known, and is suspected of containing a gene region of interest. The chromosome preparation on a slide is reacted with the probe complex, indicated at 14 in Figure 4A, and composed of a probe 16 coated with RecA protein, shown by circles at 18, and having biotin groups, indicated by vertical dashes at 26. Reaction of the probe complex with the chromosome material, in accordance with the invention, leads to homologous binding of the probe to a gene site S (Figure 4B) which is the target region of interest.

The binding site S may be visualized, for site localization by a variety of methods. In one method,

illustrated in Figure 4C, a second probe complex 22 composed of a probe 24 homologous to kn wn region 12 (gene site M) and also containing biotin groups 26 is added to the chromosome preparation, and allowed to bind 5 to its region of homology. After washing to remove unbound probe, the preparation is reacted with an FITC-avidin reporter 28, to label both sites on the chromosome with a fluorescent tag.

When viewed by fluorescence microscopy, a field such 10 as shown at Figure 4C is seen, with the two fluorescence points, shown at 30 in Figure 4C, providing an indication of the distance between the marker and test sequences on the chromosomes.

In another visualization method, shown in Figure 4D, 15 the chromosomes are labeled with one or more specific fluorescent dyes, indicated at 32, which give characteristic staining patterns in metaphase chromosomes (Korenberg, Lawrence, 1990). The chromosomes are also labeled with an avidin reporter 34 containing a 20 fluorescent label having a different fluorescence excitation wavelength from that of the band staining fluorescent molecule(s). Using fluorescence microscopy, the chromosomes are visualized at one wavelength, as indicated at 36 in Figure 4D, and the location of the probe on the chromosomes site is visualized at a second 25 excitation wavelength. Although reaction with one homologue is shown (4D) all homologous sequences would react with probe.

The invention also provides an improved method for 30 detecting a variety of chromosomal abnormalities.

Figures 5-10 illustrate how the method can be applied to detecting various types of chromosome 35 aberrations. Figure 5, frame A shows a normal chromosome 40a containing two linked marker regions 42 and 44 on one of the chromosome arms. The two regions in the

chromosome are hybridized with individual probe complexes, in accordance with the invention, then labeled with different fluorescent tags. For example, one of the regions may be labeled with an avidin-linked fluorescence reporter specific against biotin groups on one probe complex, and the second region, labeled with a second fluorescence reporter carried on an anti-digoxigenin antibody specific against digoxigenin groups on the second probe complex. The first and second fluorescence reporters are indicated by open and solid circles, respectively in Figure 5 and in related Figures 6-10.

When the two regions are examined by fluorescence microscopy, at the appropriate excitation wavelengths, the two regions are localized by two distinguishable fluorescence spots (indicated by open and solid circles, in frame B). The two spots indicate the relative orientation of and distance between the two genomic regions in the normal chromosome.

Figure 6 illustrates, in frame A, a chromosome 40b which differs from chromosome 40a by a deletion of chromosome region 44. The mutation is seen, in frame B, as a single fluorescence spot at an excitation wavelength corresponding to region 42 only.

Figure 7 illustrates, in frame A, a chromosome 40c which differs from chromosome 40a by an insertion between regions 42, 44 in the chromosome. The insertion is evidenced, in the fluorescence microscopy field seen in frame B, by a greater distance between the two fluorescence spots with respect to the Figure 5 distance.

Figure 8 illustrates, in frame A, a chromosome 40d which differs from chromosome 40a by a duplication of the region 44. The duplication is seen, in Frame B, as a doublet at the excitation wavelength of the region 44 probe, as indicated.

Figure 9 illustrates, in Frame A, a chromosome 40e

which differs from chromosome 40a in that a segment containing region 44 has translocated to a second chromosome 48e. The translocation is evidenced, in Frame B, by widely spaced fluorescence spots. The identity of chromosome 48e may be determined, as above, by staining the chromosomes with dyes which form characteristic metaphase banding patterns (or using chromosome 48 marker hybridization), as above.

Figure 10 shows, in frame A, a chromosome 40f which differs from chromosome 40a in that the segment carrying regions 42, 44 has been inverted. The inversion is evidenced, in Frame B, by reversal of positions of the two fluorescence spots.

Figure 12 shows the ability of the method of the present invention to detect specific chromosomal DNA sequences in metaphase chromosomes using native RecA-mediated fluorescence *in situ* hybridization. These data support the use of the method of the present invention for native fluorescence *in situ* hybridization on slides. Example 6 describes the steps used to generate the metaphase chromosome fluorescence *in situ* hybridization signals represented in Figure 12 including the following: the preparation of chromosome 1 alpha-satellite probe and HEp-2 cells pretreated with acetate buffer at 60°C. As expected, in Figure 12, the FITC hybridization signal is located at the centromere. These data support that the native RecA-mediated fluorescence *in situ* hybridization technique can be used to visualize sequence and gene position on nondenatured DNA in fixed chromosomes or chromatin.

Figures 13 and 14 show the ability of RecA-mediated native fluorescence *in situ* hybridization detection of tumor suppressor gene sequences. The native RecA-mediated fluorescence *in situ* hybridization technique can be used to detect and visualize a unique single copy gene

sequence in fixed cells in suspension (Figures 13A to 13F) and on slides (Figures 14A to 14D) without any signal amplification steps. The results show the detection of unique p53 sequences on chromosome 17 in 5 ATCC HEp-2 and HCC "Alexander" cells (Examples 7 and 8). Figure 15 illustrates the ability of RecA-mediated native fluorescence *in situ* hybridization to detect HBV nucleic acid sequences in ATCC HCC "Alexander" cells in suspension. Figure 15 (Example 9) shows hybridization 10 signals obtained using two different biotinylated HBV probes, "BIOPROBE" (Figure 15A to 15B) and pAM6 (Figure 15C to 15E). Viral targets were detected in ATCC HCC "Alexander" cells, known to contain HBV nucleic acid sequences, probed using the native fluorescence *in situ* 15 hybridization technique in cell suspension. Hep-2 cells, not infected with HBV nucleic acid sequences and probed with the same probes and techniques, did not show any hybridization signals. These results support the use of the method of the present invention to detect 20 diagnostically important viral target sequences in HBV-infected human liver cells.

Figure 16 demonstrates the specificity of HBV target detection using native fluorescence *in situ* hybridization. The native fluorescence *in situ* 25 hybridization assay specifically identifies nucleic acid targets homologous to probe DNA (Figure 16 and Table 1). This was demonstrated by showing that biotinylated pAM6 HBV DNA probe hybridization signal is specifically 30 competed when reactions contain excess homologous unlabeled pAM6 DNA (Figure 16A) but not when they contain either excess nonhomologous unlabeled  $\phi$ X174 DNA (Figure 16B) or excess unlabeled human placenta DNA (Figure 16C). The results of these competition experiments demonstrate 35 that native RecA-mediated fluorescence *in situ* hybridization signals, e.g., with HBV probe DNA and HCC

cells in suspension, are HBV specific.

Generally, the RecA-mediated fluorescence *in situ* hybridization reactions of the present invention use RecA protein, cofactor, and 1-2 hour incubation times.

5 Single-stranded probes in a broad size range work, including, but not limited to, average sizes of 100-200, 200-400, 300-500, 400-600, and up. Typically, size ranges above 100-200 are preferred and 300-500 are most preferred. Probes coated with RecA protein can be stored 10 in the freezer for future use: probes stored for up to 7 days have been tested and gave good hybridization signals.

15 The above described competition experiments have demonstrated that the RecA-mediated native fluorescence *in situ* hybridization is specific for detecting homologous nucleic acid sequences. The hybridization reaction is capable of detecting single copy genes and sequences (e.g., p53), multiple copy sequences (e.g., alpha-satellite chromosome 1), and diagnostically 20 important viral target sequences (e.g., HBV). Native RecA-protein mediated fluorescence *in situ* hybridization reactions are in general, more rapid than standard denatured fluorescence *in situ* hybridization assays. Experiments performed in support of the present invention 25 indicate that washing in 1.75X SSC after hybridization improves signal and decreases background.

30 Some features of the present invention for native RecA-mediated fluorescence *in situ* hybridization include the following: native RecA-mediated fluorescence *in situ* hybridization can be used on 1X PBS washed, 100% methanol fixed (or 70% ethanol fixed) cells in suspension; signals can be achieved with two hours, or less, of incubation with probe; the reaction is efficient -- for example, with 50ng probe and standard conditions, the reaction 35 averages between 65-90% of cells with signal, depending

upon the concentration of probe used; the reaction works with less than 50ng probe -- concentrations of probe in excess of 10 ng are preferred; a number of cofactors, including ATP $\gamma$ S, GTP $\gamma$ S, ATP, dATP and a combination of ATP $\gamma$ S and ADP, work in these reactions -- one embodiment employs ATP $\gamma$ S concentrations in the range of approximately 0.24 to approximately 2.4 mM (preferred embodiments include the range of approximately 0.24 to 0.48 mM); a wide range of RecA monomer:nucleotide ratios work well, including 1:1, 1:0.8, 1:2 and 1:2.5 (a preferred embodiment utilizes 1:2); the amount of signal obtained with a Chromosome #1 alpha-satellite probe and native RecA-mediated fluorescence *in situ* hybridization on slides with HEp-2 cells are comparable to those obtained using a standard denatured fluorescence *in situ* hybridization technique; the reaction works in the presence of accessory proteins (e.g., single-strand binding protein (SSB), topoisomerase I and topoisomerase II); and when the reactions are carried out for samples fixed on slides the reaction efficiency is improved from an average range of 5-20% to 55-80%, by incubating slides in 10mM Tris-acetate buffer pH7.5 at 55-60°C for 30-45 min before adding RecA-coated probe mix. This temperature is below the denaturation temperature of intracellular nucleic acids.

It will be appreciated that the above applications of the method, to the extent they involve probe binding to a single or small-copy-number target sequence are uniquely suited to study by the present method.

Another general application of the method of the invention is for diagnostics, typically for detecting changes in chromosome ploidy or rearrangement, or presence of a viral or bacterial or parasitic pathogen in an infected organism, organ, tissue, or cell. This application is specifically discussed above and is

generally illustrated in Figures 11A-11C for detection of virus infected cells, such as cell 50. Virion particles (or integrated viral genomes) contained in the cell are shown at 54. The cells, e.g., blood cells, are obtained from the test subject, and treated to permeabilize the cellular structures, as discussed above. To the permeabilized cells (Figure 11A) is added a virus-specific DNA probe complex 56, with sequence specific binding of the DNA complex to virus duplex nucleic acid being followed by addition of a fluorescent marker molecule 58, for virus-complex labeling (Figure 11B). The probe signal may be enhanced, if necessary, by the amplification of reporter reagents described above, e.g., a biotinylated anti-avidin antibody, followed by a second 15 fluorescence-labeled avidin reporter molecule.

The labeled cells may be examined by fluorescence microscopy, to detect and localize infecting virus nucleic acid in the cells. Alternatively, cell infection, and percent cells infected, can be determined by fluorescence activated cell sorting (FACS), as illustrated in Figure 11C. This figure shows a group of blood cells, such as cells 60, 62 passing through a capillary tube 64 in a FACS device equipped with a detector 66 for detecting fluorescence in individual cells passing through the detector region. Fluorescence labeled cells are indicated by dark shading in the figure. It is seen that the method provides rapid detection of infected cells, for diagnostic purposes, and is capable of measuring level of infection and percentage of cells infected. Thus, for example, the method can be used to assess the progress of an anti-virus treatment, by measuring decreases in cell infection over the treatment period.

The FACS device may be further equipped with sorting apparatus for capturing fluorescence-labeled cells, to

form a concentrate of infected cells. The concentrate, in turn, can be used as a source of viral nucleic acid, for purposes of identifying and cloning the viral genome.

5 The following examples, which are intended to illustrate but not limit the invention, illustrate particular methods and applications of the invention.

Example 1

10 Purification of RecA Proteins

RecA and RecA803 proteins were isolated from the overproducing strains JC12772 and JC15369 (obtained from A.J. Clark and M. Madiraju), or RecA was purchased from Pharmacia.

15 RecA and RecA803 proteins were purified by modification of published procedures (Shibata, Griffith) involving fast protein liquid chromatography (FPLC) using a hydroxylapatite column (obtained as powder from BioRad) followed by an anion ("MONO Q", Pharmacia) exchange column.

Protein purification was monitored as follows:

(i) identifying the 38,000-dalton RecA protein by SDS-PAGE ("PHASTGEL" system, Pharmacia, Piscataway NJ);  
25 (ii) assay of the RecA ssDNA-dependent ATPase activity using [ $\gamma$ -<sup>32</sup>P]ATP and single-stranded DNA (Shibata). The products of the reaction were separated using PEI cellulose thin-layer chromatography (EM Science, NJ): the PEI plates were developed in a solvent of 0.5 M LiCl and 0.25 M formic acid. Products were detected by autoradiography.

30 (iii) assay of DNase activity. DNase activity was monitored by incubating the RecA protein samples with a mixture of  $\phi$ X174 linearized and supercoiled circular double-stranded RF and circular single-stranded DNAs in

RecA strand-transfer buffer (Cheng) for 1 hr at 37°C. DNA nicking and digestion were monitored after deproteinization by visualizing the DNAs with ethidium bromide after agarose gel electrophoresis and comparing the quantities of each DNA type in the RecA incubated samples with those incubated in buffer without RecA. Only RecA protein samples showing no detectable DNase activity were used.

5 (iv) assay of D-loop activity with 500-mer oligonucleotide probe using a method modified from Cheng. Silver stained SDS-polyacrylamide gel profiles of the final "MONO-Q"-purified RecA and RecA803 proteins showed a single 38,000-dalton band from each preparation that was essentially free of other cellular polypeptides.

10 15

Example 2

Preparation of Probe Complex

Biotinylated chromosome X alpha satellite DNA probe was obtained from ONCOR (Gaithersburg, MD).

20 Probe diluted in sterile MilliQ (Millipore) H<sub>2</sub>O was denatured in a 0.5 ml microcentrifuge tube in a 100°C heat block for 5 min, and the tube immediately placed in an ice water bath. Approximately 5 min prior to addition of denatured probe to the hybridization mixture the tube containing the probe was placed in ice in a freezer at -20°C. The probe hybridization mixture contains the following components in a broad range of concentrations and is combined in the order listed: 1  $\mu$ l of 10X RecA reaction buffer (10X RecA reaction buffer: 100mM Tris acetate pH 7.5 at 37°C, 20 mM magnesium acetate, 500 mM sodium acetate, 10 mM DTT and 50% glycerol (Cheng)); 1.5  $\mu$ l ATP $\gamma$ S from 16.2 mM stock (3.24 and 1.62 mM stocks can also be used), (Pharmacia) (rATP, dATP, GTP $\gamma$ S, or a combination of ATP $\gamma$ S and ADP may be

25 30

used in some reactions); 0.75  $\mu$ l 20 mM magnesium acetate; 4-60 ng (or more in some reactions) of denatured probe in sterile ddH<sub>2</sub>O or TE (20 mM Tris HCl, pH7.5, and 0.1 mM EDTA); RecA (when prepared in our own laboratory and the exact amount of  $\mu$ l added varies depending on concentration of stock; when purchased from Pharmacia, 1.25  $\mu$ l 0.137 mM stock). The mixture was incubated at 37°C for 10 min followed by addition of 0.5  $\mu$ l/reaction of 200 mM magnesium acetate. Final concentrations of reaction components are: 4.0 mM to 10 mM Tris acetate, 2.0 mM to 15 mM magnesium acetate, 20.0 mM to 50 mM sodium acetate, 0.4 mM to 1.0 mM DTT, 2% to 5% glycerol, 0.24 mM to 2.5 mM ATP $\gamma$ S, 0.005 mM to 0.02 mM RecA.

15

### Example 3

#### In situ Hybridization with Chromosome X Probe

##### A. Preparation of HEp-2 Cell Nuclei

HEp-2 cells were originally derived from human male larynx epidermoid carcinoma tissue. HEp-2 is chromosome 20 ploidy variable (Chen).

The cells were cultured for 24 hours after seeding in DMEM (Whittaker or GIBCO-BRL) supplemented with 10% FBS, sodium pyruvate and Penstrep antibiotics mix at 37°C under standard conditions. The cells were pelleted by 25 low-speed centrifugation and gradually resuspended in 75 mM KCl in a 37°C water bath, and allowed to incubate for between 5 and 15 min for the desired amount of nuclear swelling to occur, followed by addition of 3:1 ice cold methanol:acetic acid and centrifugation at 6°C.

30 One ml of fluid was left in the tube with the pelleted cells, additional ice cold methanol:acetic acid was added, and the cells suspended by gentle mixing of the tube, followed by centrifugation. Repeated additions of methanol:acetic acid degrades cytoplasm and isolated

nuclei were stained by repeated additions of methanol:acetic acid followed by mixing and centrifugation as above. (HEp-2 and other cell types may be fixed in alternative ways, some of which do not 5 degrade fixed cytoplasmic structures).

Finally, the preparation of nuclei was resuspended in 3:1 methanol:acetic acid at a concentration about  $2 \times 10^6$ /ml and is either dropped by pipette in 10  $\mu$ l aliquots onto clean glass slides which were stored at -20°C, or 10 the suspended nuclei or cell preparation are stored at -20°C for later use.

#### B. Nondenatured Nucleic Acid Target-Hybridization Reaction

15 Ten  $\mu$ l of probe mixture/reaction from Example 2 was applied to the fixed preparation on glass slides. Glass coverslips were placed over the hybridization areas and sealed with rubber cement, and reactions were incubated enclosed in a moist container in a 37°C CO<sub>2</sub> incubator for 20 between 1-4 hours. Following incubation, the rubber cement was removed and the slides were washed in coplin jars 3 times for 10 min each in 2X SSC (20X SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0 is used in all SSC 25 containing preparations in these assays) in a water bath at 37°C. Other wash conditions may also be used.

The slides were placed in preblock solution [4X SSC, 0.1% Triton X-100, 5% Carnation nonfat dry milk, 2% normal goat serum (Gibco), 0.02% sodium azide, pH 7.0] for 25 min at room temperature (RT), followed by 30 immersion in 5  $\mu$ g/ml FITC-avidin DCS, cell sorter grade (Vector, A-2011) in preblock solution for 25 min at RT. The slides were washed in 4X SSC, 4X SSC and 0.1% Triton X-100, and 4X SSC for 10 min each at RT, followed by brief rinsing in double-distilled H<sub>2</sub>O and dried. Antifade

was applied [100 mg p-phenylenediamine dihydrochloride (Sigma P1519) in 10 ml PBS adjusted to pH 8 with 0.5 M carbonate-bicarbonate buffer (0.42 g NaHCO<sub>3</sub> adjusted to pH 9 with NaOH in 10 ml ddH<sub>2</sub>O) added to 90 ml glycerol, and 5 0.22 µm filtered], and antifade mounting medium and coverslips were placed over the preparations. Antifade containing a counterstain such as propidium iodide or DAPI was sometimes used instead of antifade alone. Figure 1A shows a fluorescence micrograph of a cell 10 nucleus from the above preparation (no signal amplification).

If necessary, signal amplification may be performed as follows: Slides are washed for 5-10 min in 4X SSC and 0.1% Triton X-100 at RT to remove coverslips and 15 antifade, followed by incubation in preblock solution for up to 20 min, then are incubated with biotinylated goat anti-avidin antibody (Vector BA-0300) at a concentration of 5 µg/ml diluted in preblock solution for 30 min at 37°C. Slides are washed for 10 min each in 4X SSC, 4X 20 SSC and 0.1% Triton X-100, 4X SSC at RT followed by incubation in preblock solution for 20 min at RT, then immersed in preblock solution with 5 µg/ml FITC-avidin for 20 min at RT. Slides are again washed in the 4X SSC series, briefly rinsed in dd H<sub>2</sub>O, and mounted with 25 antifade or antifade with counterstain.

#### C. Hybridization by Heat Denaturation of the Nucleic Acid Target.

For comparative purposes, *in situ* hybridization by 30 heat denaturation of nuclear substrate was performed in parallel. Denatured labeled X chromosome probe was added to the nuclei, denatured on a slide under ONCOR protocols. The same nuclear preparations were used as in the nondenatured method. The signal amplification

procedure suggested by ONCOR was used to enhance the hybridization signal. Thereafter, the slide was maintained at 37°C overnight. The procedures and materials generally followed that of the ONCOR Chromosome

5      In situ Kit, Cat No. S1370.

Figure 1B shows a fluorescence micrograph of a cell nucleus from the above signal amplified preparation.

Example 4

10      In situ Hybridization with Chromosome 7 Probe

Biotinylated DNA probe to chromosome 7 alpha satellite DNA was obtained from ONCOR. The probe was denatured and could be stored frozen for at least five weeks. 32 ng of denatured freshly thawed DNA probe in 16  
15      ul (1:2, probe:H<sub>2</sub>O, 2 ng/ul DNA) were added to the same amount of hybridization mixture and in the same order given in Example 2. Following incubation of the probe mixture at 37°C for 10 min and final addition of 0.5 ul 200 mM magnesium acetate, the reaction contained a total  
20      of 21 ul.

Probe was incubated on the nondenatured HEp-2 target cell nuclei (Example 3B) for 2.5 hours at 37°C in a CO<sub>2</sub> incubator followed by washing, blocking, and FITC-avidin incubation exactly as described for probe to chromosome X in Example 3B. The time to conduct the experiment, including the ethanol series treatment of the slide was approximately 5 hours. Figure 2A shows a fluorescence micrograph of a cell nucleus from the treated preparation.

30      For comparison, the nuclei were reacted with chromosome 7 probe under heat-denaturation conditions, as in Example 3C. Briefly, 5 ng denatured probe to chromosome 7 alpha satellite DNA was combined with hybridization buffer (Hybris 1 V1, ONCOR, as in Figure

1B) and denatured using ONCOR protocols. 7  $\mu$ l of the probe mixture was hybridized with HEp-2 cell nuclei for 16 hours and the reaction treated according to ONCOR protocols, including signal amplification. Figure 2B shows a fluorescence photomicrograph of the treated denatured nuclei.

Example 5

10 Detection of Specific Chromosome Sequences  
in Methanol Fixed Interphase Nuclei

### in Suspension

A probe specific for the X chromosome alpha satellite DNA, Oncor probe stock (also used in Example 2) was diluted and denatured at 100°C for 5 min, immediately placed in an ice-water bath (for approximately 15 min) and stored in a -20°C freezer briefly (about 5 min) before addition to the hybridization mixture. The hybridization mixture was combined in the following order (components, concentrations, and mixtures are described in detail in Example 2): 1  $\mu$ l 10X RecA reaction buffer (see Example 2), 1.5  $\mu$ l ATPyS (16.2 mM stock, Pharmacia), 0.75  $\mu$ l magnesium acetate (20 mM stock), 12  $\mu$ l of denatured probe (ONCOR) containing 60 ng in a 1:2 dilution in  $H_2O$  (20 ng or more than 60 ng can also be used), RecA (0.137 mM stock, Pharmacia). The mixture was incubated in a 37°C water bath for 10 min followed by addition of 0.5  $\mu$ l 200 mM magnesium acetate.

HEp-2 cells were fixed in 100% methanol- (or other appropriate solutions) at -20°C at a concentration of approximately  $2.5 \times 10^6$ /ml. About 0.5 ml of the suspended cells ( $1.25 \times 10^6$ ) were centrifuged in a "TOMY" centrifuge set at 6°C in a 1.5 ml microcentrifuge tube and resuspended followed by centrifugation in 200  $\mu$ l to 1 ml

of 70%, 85% and 100% ice cold ETOH. After the final centrifugation and removal of 100% ETOH supernatant the pellet was resuspended in 200-500  $\mu$ l 1X RecA reaction buffer at RT, and placed in a 0.5 ml centrifuge tube and centrifuged.

5 The completed probe mixture was mixed with the pellet, and the tube placed in a 37°C water bath for 1.5-2.5 hours. Incubation was stopped by addition of 250  $\mu$ l 2 X SSC (prewarmed to 37°C) followed by centrifugation.

10 The pellet was resuspended in 2 X SSC (prewarmed to 37°C) and incubated for 5 min at 37°C. Following centrifugation the pellet was resuspended in 500  $\mu$ l blocking solution at RT for 20 min, then centrifuged and resuspended in 10  $\mu$ g/ml FITC-avidin in 100  $\mu$ l blocking

15 solution at RT in the dark, for 20 min. The tube was centrifuged and 250  $\mu$ l 4 X SSC mixed with the pellet, again centrifuged, and 250  $\mu$ l 4 X SSC with 0.1% Triton X-100 mixed with the pellet and again centrifuged with 250  $\mu$ l 4 X SSC all at room temperature. After a final

20 centrifugation the pellet was mixed with approximately 20  $\mu$ l antifade. Specific signal was noted in approximately 30% of the suspended cells. Note: Experiments using 100% methanol fixed whole cells and/or fixed nuclei and other concentrations of different washing components have

25 shown 50-90% reaction.

The Figure 3A photomicrograph shows a dividing fixed HEp-2 cell nucleus, as viewed with a Zeiss LSM-10 microscope, illustrating the symmetrically located FITC-labeled probe-bound centromeric targets. The phase picture in Figure 3B below was taken of the same nucleus without changing the microscope focus.

Example 6

35 Detection of Specific Chromosomal DNA Sequences  
in Metaphase Chromosomes

Bi tinylat d pr be to chr mos m '1 alpha-satellite centromeric sequences (pUC1.77: a 1.77 base pair long human EcoRI fragment in the DNA vector pUC9; Cooke, et al.; Emmerich, et al.) was prepared using the BRL 5 Nick-translation system in the presence of bio-14-dATP (Gibco-BRL, Gaithersburg MD). The nick translations were performed essentially as described by the manufacturer (BRL) with the following modification: twice the recommended amount of enzyme was added and the reaction 10 was incubated at 15°C for 1hr 45 minutes. These nick translation reaction resulted in probes with an average single-strand size of approximately 300-400 bp.

Nick-translated probes were precipitated in 0.3M sodium acetate in ethanol, resuspended in 10 mM Tris-HCl 15 pH 7.5, 0.1 mM EDTA, and the DNA concentration was determined with the "DNA DIPSTICK" (Invitrogen). Methanol:acetic acid fixed HEp-2 cells (mostly nuclei; prepared similarly to Example 3) mounted on slides were dehydrated by exposure to a series of 70, 85, and 100% 20 cold ethanol incubations. Dehydrated cells on slides were then preincubated in 10mM Tris-acetate buffer, pH 7.5, at 60°C for 45 minutes while the RecA-coated chromosome 1 alpha-satellite centromeric sequence probe mix was prepared.

25 The 60°C preincubation treatment does not denature target DNAs but it does improve the efficiency of native RecA-mediated fluorescence *in situ* hybridization reactions performed on fixed cells on slides (from 5-20% to 60-82% improved hybridization). The warmed slide was 30 cooled to 37°C on a 37°C surface before prepared probe mix was added to the fixed cell nuclei preparation. Cells were covered with a coverslip and the reaction was sealed with rubber cement.

The DNA pr be was heat denatured at 100°C in 5.16  $\mu$ l

dd H<sub>2</sub>O for 5 minutes, quick-centrifuged in an ice-water bath, centrifuged at 4°C in a "TOMY" microcentrifuge for 20 seconds to collect the liquid, and then immediately added to a mixture containing the other reaction components.

5 Chromosome 1 probe was coated with RecA protein in a reaction mixture containing 1  $\mu$ l of 10 X acetate reaction buffer (Cheng et al, 1988), 1.5  $\mu$ l of 16.2 mM ATP $\gamma$ S (Sigma), 0.75  $\mu$ l of 20 mM MgOAc, 0.59  $\mu$ l of RecA (11.05  $\mu$ g/ $\mu$ l), 1  $\mu$ l of DNA probe (50 ng/ $\mu$ l). The total volume 10 of reaction mix after probe addition was 10  $\mu$ l. The probe reaction mix was incubated at 37°C for 10 minutes and then 0.5  $\mu$ l of 0.2 M MgOAc was added. Probe mix was then added to the buffer-treated cell nuclei on slides at 15 37°C. The reaction was covered with a coverslip, sealed with rubber cement and incubated in a moist chamber at 37°C for 2 hr.

After cell incubation with probe, the rubber cement was removed and the slide was washed 3X in 1.75X SSC (pH7.4) at 37°C, each wash was 10 minutes. The slide was 20 incubated in filtered preblock solution (100  $\mu$ l) at room temperature for 20 minutes, then with 5  $\mu$ g/ml FITC-Avidin (Vector, DCS grade) in filtered preblock at room temperature for 20 minutes in the dark.

Slides were washed at room temperature 1X in 4X SSC, 25 1X in 4X SSC + 0.1% "TRITON X-100", and then finally, in 1X in 4X SSC. Slides were dipped into ddH<sub>2</sub>O briefly after the last wash and allowed to air dry. Before coverslip addition, antifade was added and the cells were observed with a Zeiss LSM.

30 Figure 12 shows the hybridization signal from the fixed HEp-2 metaphase chromosomes with the RecA-coated, biotinylated, nick-translated probe to human chromosome 1 alpha-satellite centromeric sequences. Under these conditions, 73% of the cell interphase nuclei including

chr mos m spreads showed signals. The chr mosome 1 alpha satellite specifically hybridized with the chromosome 1 centromere.

5

Example 7RecA-Mediated Native Fluorescence In Situ HybridizationDetection of Unique p53 Chromosome 17Tumor Suppressor Gene SequencesA. First Conditions: Figures 13A and 13B.

10. 1.25 x 10<sup>6</sup> 100% methanol fixed ATCC HEp-2 (ATCC; American Type Culture Collection, 12301 Parklawn Dr., Rockville MD 20852) cells were placed in a microcentrifuge tube and put through an ethanol series of 70%, 85% and 100% (Lawrence, 1988 and 1990; Example 5).  
15. The cells were pelleted between fixation steps. After the 100% ethanol treatment step the cells were saved as a pellet until just before addition of the 1X acetate reaction buffer wash. All cell centrifugations between steps were for 30 seconds at 2.5 K in a "TOMY" microcentrifuge.  
20.

While probe is incubating with RecA protein, the pelleted cells are washed in 1X acetate reaction buffer (Cheng et al, 1988). The cells are pelleted again and as much of the buffer wash as possible was removed before 25. the addition of the RecA-coated probe reaction mix.

Probe was coated with RecA protein for 10 minutes at 37°C in a mix containing 1  $\mu$ l of 10X acetate reaction buffer, 0.75  $\mu$ l of 0.02M MgOAc, 1.5  $\mu$ l of 1.62 mM ATP $\gamma$ S (Sigma), 0.59  $\mu$ l of 11.02  $\mu$ g/ $\mu$ l RecA, heat denatured 30. probe [5  $\mu$ l p53 probe (10 ng/ $\mu$ l; Oncor Inc., Gaithersburg MD) and 1.16  $\mu$ l ddH<sub>2</sub>O]. Before probe addition to washed cell pellet, 0.5  $\mu$ l of 0.2M MgOAc was added to the probe mix. Cells were mixed with probe and incubated for 3 hr 50 minutes at 37°C.

After incubation, cells were washed 3X in 1.75X SSC pH7.4 (250  $\mu$ l washes), then incubated at room temperature for 20 minutes in filtered preblock, pelleted, and the preblock removed. This step was followed by incubation 5 at room temperature for 20 minutes with 50  $\mu$ l of filtered preblock containing 5.0  $\mu$ g/ml FITC avidin. Cells were washed in 4X SSC, 4X SSC + 0.1% "TRITON X-100", 4X SSC, all pH7.4, (250  $\mu$ l/wash).

10 A small amount (e.g., approximately 20  $\mu$ l) of antifade was added to the final cell pellet and a portion of the cells were placed on a slide, covered with a coverslip, and observed using a Zeiss LSM. Under these general conditions, 65% or more of the cells show bright p53 hybridization signals (Figures 13A and 13B).

15

B. Second Conditions: Figures 13C and 13D.

20 All cells and cell washes were identical to Example 7A. Probe was reacted with RecA as described above with the exception that the 0.02M MgOAc was omitted and 0.75  $\mu$ l of ddH<sub>2</sub>O was added instead. Under these conditions, 40% of the cells had bright hybridization signals (Figures 13C and 13D).

25

C. Third Conditions: Figures 13E and 13F.

30 All cell washes were identical to Example 7A. Probe was reacted as described above (Example 7A) with the exception that probe coating mix contained 1.5  $\mu$ l 16.2 mM ATP $\gamma$ S, the reaction mix was incubated for 13 minutes at 37°C before addition of 0.5  $\mu$ l 0.2mM MgOAc, RecA-coated probe was added to 1.25 x 10<sup>6</sup> 100% methanol fixed ATCC HCC "Alexander" cells and reacted for 3 hr 20 minutes at 37°C. Cell washing after probe reaction was as described in Example 7A except that cells were reacted with 50  $\mu$ l of filtered preblock containing 10.0  $\mu$ g/ml FITC-Avidin.

Under these conditions, 82% of the cells had bright hybridization signals (Figures 13E and 13F).

Example 8

5      RecA-mediated Native Fluorescence In Situ Hybridization  
          Detection of Unique p53 Gene Sequences in HEp-2 Cell  
          Nuclei on Slides

Methanol:acetic acid fixed ATCC HEp-2 cells on slides were reacted with RecA-coated p53 (Oncor) probe. 10 Cells were washed and prepared for probe addition as described in Example 6 with the exception that the 45 minutes incubation with 10 mM acetate buffer pH7.4 was omitted.

15      p53 probe DNA coating was done as described in Example 6 except that 1.5  $\mu$ l of 3.24 mM ATP $\gamma$ S, 0.59  $\mu$ l of 5.51  $\mu$ g/ $\mu$ l RecA and 0.5  $\mu$ l containing 2U topoisomerase II (United States Biochemicals Corp., Cleveland OH) were added, and half as much denatured probe was added [2.5  $\mu$ l (25 ng probe) in 3.66  $\mu$ l dd H<sub>2</sub>O].

20      After probe coating with RecA protein, 0.5  $\mu$ l 0.2M MgOAc was added and the probe mix was applied to nuclei on slides. Washing conditions after reaction with probe were as described for Example 6. Under these conditions, 20% of the nuclei had bright hybridization signals 25 (Figures 14A to 14D). The number of interphase nuclei with hybridization signals in this experiment is less than observed in Figure 12 (Example 6) -- no buffer incubation step was included in this protocol.

30

Example 9

RecA-Mediated Native Fluorescence In Situ Hybridization  
          Detection of HBV Nucleic Acid Sequences

in ATCC HCC "Alexander" Cells in Suspension

1  $\times$  10<sup>6</sup> of 100% methanol fixed HCC cells/reaction are

placed in 0.5 ml sterile microfuge tubes, centrifuged for 30 seconds at 2 K rpm in a "TOMY" microcentrifuge at 4°C, and the supernatant removed. 200  $\mu$ l of ice-cold 70% EtOH is added, the treated cells are centrifuged at 4°C, the supernatant removed, the dehydration step repeated and the sample centrifuged as above using, sequentially, 85% and 100% iced-cold EtOH.

5 The cells are centrifuged and resuspended in 200  $\mu$ l 1X acetate reaction buffer (same as standard RecA acetate reaction buffer except, minus the glycerol), centrifuged, and resuspended in same 1X acetate reaction buffer (minus glycerol). Immediately before the addition of the probe reaction mixture, the cells are incubated at 37°C for 10 minutes, centrifuged at room temperature and the supernatant removed.

10 Biotin-labeled HBV-specific "BIOPROBE" was obtained from Enzo Diagnostics, Inc. (New York NY). This nick-translated probe is biotinylated with bio-11-dUTP, contains the whole HBV genome (adr4 serotype) and double-stranded probe fragments average 250 bp in size.

15 A second probe, pAM6, was obtained from the ATCC. pAM6 contains the whole HBV genome (adw serotype) in plasmid pBR322. pAM6 was labeled with bio-14-dATP by nick-translation with the BRL Nick-translation System as described in Example 6. Heat denatured single-stranded probe averaged 300-500 bases in size.

20 Both HBV probes were coated with RecA protein a 10  $\mu$ l reaction containing 1  $\mu$ l 10X acetate reaction buffer (Cheng, et al, 1988), 1.5  $\mu$ l 3.24 mM ATP $\gamma$ S, 0.75  $\mu$ l 20 mM MgOAc, 0.53  $\mu$ l 5.5  $\mu$ g/ $\mu$ l RecA, and heat denatured probe [0.83  $\mu$ l "BIOPROBE" (60  $\mu$ g/ $\mu$ l) was in 5.39  $\mu$ l ddH<sub>2</sub>O; 5  $\mu$ l pAM6 probe (10  $\mu$ g/ $\mu$ l) was in 1.22  $\mu$ l ddH<sub>2</sub>O]. Probe coating reactions were incubated at 37°C for 10 minutes, then 0.5  $\mu$ l of 0.2M MgOAc stock solution was added and

the pr be mixes w re add d to the prepar d cell pellets.

The prepared probe mixes were individually added to separate cell samples and incubated at 37°C in waterbath 5 for 2 hours. The reaction was stopped by the addition of 250 µl 1.75X SSC (pH 7.4) at 37°C. Each sample was mixed, the cells pelleted and the supernatant removed. 10 250 µl of 1.75X SSC was added and the samples incubated at 37°C for 5 minutes. This wash was then repeated. The cells were pelleted and to each sample 300 µl of filtered preblock was added. The samples were incubated at room temperature for 20 minutes. The cells were pelleted and the preblock removed.

To the samples 90 µl of 5 µg/ml FITC-Avidin in 15 filtered preblock was added. The samples were incubated at room temperature in the dark for 20 minutes. The samples were then pelleted and the supernatant removed. To each sample 250 µl 4X SSC was added, the sample mixed gently, and the cells pelleted. The supernatant was 20 removed and 250 µl 4X SSC + 0.1% "TRITON X-100" added. Pellet cells, remove supernatant, add 250 µl 4X SSC. The cells were pelleted, supernatant removed, the pellet air dried, and 20 µl of antifade added. The samples were then examined using a Zeiss LSM.

25 Figures 15A and 15B show the results of the above hybridizations using "BIOPROBE": 81% of the cells had hybridization signals. Figures 15C to 15E show the results of the above hybridizations using the pAM6 probe: 95% of the cells had hybridization signals.

30

#### Example 10

Specificity of HBV Target Detection  
Using Native Fluorescence In Situ Hybridization  
in Human HCC Cells Tested by Competition Hybridization

35 A. Preparation of probes for competition assay.

Both biotinylated and unlabeled pAM6 (ATCC) and φX174 RFI (N W England Biolabs) DNAs were prepared by nick-translation using the BRL Nick-translation System. Nick-translation was carried out essentially as described in Example 6, except that reactions for producing unlabeled DNAs contained dATP in place of bio-14-dATP.

Each competition reaction used 1 X 10<sup>6</sup> 100% methanol fixed cells and contained 30 ng of biotinylated pAM6 HBV probe DNA and 240 ng of competitor DNA. Biotinylated HBV probe DNA and unlabeled competitor DNAs were coated with RecA in separate reactions. After RecA coating, the Mg<sup>++</sup> ion concentration of each reaction was adjusted by adding 0.5  $\mu$ l of 0.2mM MgOAc per 10  $\mu$ l of coating reaction. Then 10.5  $\mu$ l of RecA-coated bio-pAM6 probe (30 ng of DNA) was mixed with an equal volume of RecA-coated competitor DNA (240 ng). The final volume of each mixture of RecA-coated biotinylated HBV probe and competitor DNA was 21  $\mu$ l.

All biotinylated pAM6 DNA was coated with RecA and prepared for use in a single reaction, 10.5  $\mu$ l of which was used for each competition experiment. Coating of all the biotinylated pAM6 probe in one reaction insured that there were no differences between reactions other than the DNA competitors. To allow proper RecA coating, both probe and competitor DNA coating reactions contained the same average RecA to nucleotide ratio (1 RecA protein monomer:2 nucleotides).

All the biotinylated pAM6 probe was coated with RecA in a reaction containing 4  $\mu$ l of 10X acetate reaction buffer (Cheng, et al, 1988), 6  $\mu$ l of 3.24 mM ATP $\gamma$ S, 3  $\mu$ l of 20 mM MgOAc, 3.16  $\mu$ l of 2.2  $\mu$ g/ $\mu$ l RecA, and 12  $\mu$ l of 10 ng/ $\mu$ l bio-pAM6 probe (which was heat denatured in 11.84  $\mu$ l ddH<sub>2</sub>O).

Each competitor RecA DNA probe coating mix contained

1  $\mu$ l of 10X acetate reaction buffer, 1.5  $\mu$ l 3.24 mM ATP $\gamma$ S, 0.75  $\mu$ l 20 mM MgOAc, 1.25  $\mu$ l 11.05  $\mu$ g/ $\mu$ l RecA, and either 4.8  $\mu$ l of 50 ng/ $\mu$ l competitor DNA heat denatured in 0.7  $\mu$ l ddH<sub>2</sub>O (non-biotinylated  $\phi$ X174 or non-  
5 biotinylated pAM6), or 2.4  $\mu$ l of 5  $\mu$ g/ $\mu$ l non-biotinylated  
placenta DNA ("BLOCKIT"; Oncor) heat denatured in 3.1  $\mu$ l  
ddH<sub>2</sub>O.

All probes were heat denatured at 100°C for 5 minutes, cooled in ice-water approximately 20 sec, spun  
10 in a 4°C microcentrifuge to collect all the liquid and immediately added to their respective RecA reaction mixture.

Probes were coated with RecA for 15 minutes at 37°C and then 0.5  $\mu$ l of 0.2M MgOAc was added/10  $\mu$ l DNA  
15 mixture.

#### B. Reaction Mixtures.

The -20°C stored methanol-fixed cells were prepared for fluorescence *in situ* hybridization as previously described in Example 9 by dehydrating through a series of cold EtOH washes, followed by 2 times washes in 1X acetate reaction buffer (minus glycerol). Cells were incubated in the last wash buffer for 10 minutes at 37°C before buffer was removed and the 21  $\mu$ l of RecA-coated  
20 biotinylated probe and competitor DNA mixtures were added to the cell pellets.

Probes were reacted with cells in a 37°C water bath for 3 hrs. Reactions were stopped by addition of 250  $\mu$ l 1.75X SSC (pH7.4) at 37°C, mixed, centrifuged at room  
30 temperature (R T) to pellet cells, and supernatant removed. Cells were washed twice with 250  $\mu$ l 1.75X SSC at 37°C for 5 minutes then spun down and the supernatant removed.

300  $\mu$ l filtered preblock was added to treated,

washed cells and incubated at RT for 20 minutes. After centrifugation and supernatant removal, 90  $\mu$ l of 5  $\mu$ g/ml FITC-Avidin in filtered preblock was added to each reaction, incubated at room temperature for 20 minutes in the dark. FITC-Avidin was removed after cells were pelleted by centrifugation. Reacted cells were washed consecutively in 4X SSC (pH7.4) mixed gently with the cells, 250  $\mu$ l 4X SSC + 0.1% "TRITON X-100" and 250  $\mu$ l 4X SSC. After each wash, cells were pelleted and the wash liquid removed.

After the final wash, the cells were air dried and approximately 20  $\mu$ l of antifade was added to each cell reaction. Cells were mounted on slides, covered with a coverslip and examined with the Zeiss LSM. Cells containing moderate to bright hybridization signal(s) were scored as positive for hybridization (see Table 1).

Table 1

Specificity of HBV fluorescence *in situ* hybridization  
in human HCC cells

Competing DNA <sup>a</sup>	#Cells with strong FITC FISH* Hybridization Signal	# Cells Counted	% Cells with strong FITC FISH Hybridization Signal
HBV	0	154	0 <sup>b</sup>
$\phi$ X174	39	105	37.1
Placenta	30	99	30.3

25 Nonbiotinylated.

30 <sup>b</sup>4.5% of these cells showed very faint FITC hybridization. Whereas FITC signals with the other competing DNAs were easily visible using the fluorescence microscope alone, the signals with this sample were only visible when 488 nm argon-ion laser illumination was used.

35 \* fluorescence *in situ* hybridization.

The results presented in Table 1 show that only

homologous HBV DNA, not heterologous DNAs, specifically competes with the biotinylated HBV DNA probe signal.

The cells shown in Figures 16A to 16C are from the competition experiments described in Table 1. In Figure 5 16: 16A, Biotinylated HBV probe + excess unlabeled HBV probe DNA; 16B, Biotinylated HBV probe + excess unlabeled φX174 DNA; 16C, Biotinylated HBV probe + excess unlabeled human placenta DNA ("BLOCKIT"; Oncor). FITC probe signals were observed with a Zeiss LSM in laser scanning 10 mode.

The observed FITC signals from the HBV probes are shown superimposed on the phase images of the cells. Several cells from each experiment are shown. It is clear from the signal and cell images that homologous HBV 15 DNA specifically competes with the biotinylated HBV DNA probe signal but heterologous DNA does not compete.

Although the invention has been described with 20 respect to particular protocols and applications, it will be appreciated that a variety of changes and modifications may be made without departing from the invention.

## IT IS CLAIMED:

1. A method of identifying the presence of a known target sequence in a double-stranded nucleic acid contained in a fixed cellular or subcellular biological structure, in a defined morphological relationship with the structure, comprising

5 adding to the structure, a probe complex composed of RecA protein stably bound to a single-stranded, reporter-labeled probe which is complementary to a duplex target sequence, under conditions in which the complex can contact the duplex nucleic target,

10 allowing the complex to bind to the target sequence under non-denaturing conditions,

15 removing unbound complex from said structure, and examining the structure for the presence of the reporter-labeled probe bound to the nucleic acid.

2. The method of claim 1, wherein the complex is stabilized by the presence of a cofactor selected from the group consisting of ATP $\gamma$ S, GTP $\gamma$ S, ATP, dATP and a combination of ATP $\gamma$ S and ADP.

3. The method of claim 1, wherein said probe is labeled with a ligand reporter, and said examining includes adding to the structure, specific ligand molecule, including antibodies, effective to stably bind to said ligand, and having a detectable reporter group.

30 4. The method of claim 1, for detecting the presence in a host cell, of a pathogenic (foreign) target duplex nucleic acid sequence, wherein said complex is added to the cells under conditions of host cell fixation, and said examining includes detecting the presence of a probe-bound reporter in said fixed cells.

5. The method of claim 1, wherein said examining includes detecting a fluorescent reporter bound to the reporter-labeled probe bound to the nucleic acid using either microscopy or a fluorescence activated cell sorter.

10 6. The method of claim 1, for localizing a selected target duplex nucleic sequence integrated into a host-cell genome, wherein said complex is added to the chromosomes of the cell, and said examining includes examining the chromosomes microscopically to determine the relative position of reporter-labeled probe in relation to chromosome ultrastructure.

15 7. The method of claim 6, wherein said chromosomes are labeled with one fluorescence reporter, said probe is labeled with a second fluorescence reporter, and said examining includes viewing the cells by fluorescence microscopy separately at wavelengths effective to excite fluorescence in each of the two reporters.

20 8. The method of claim 6, for localizing the target sequence in a selected chromosome, which further includes adding to the structure a second probe complex composed of RecA protein stably bound to a single-stranded, reporter-labeled nucleic acid probe which is complementary to a duplex strand in a known region of the selected chromosome, and said examining includes determining the relative positions of reporters associated with each of the two complexes.

25 9. The method of claim 8, wherein the first-mentioned complex and the second complex are labeled with different fluorescence reporters, and said examining includes viewing the cells by fluorescence microscopy

separately at wavelengths effective to excite fluorescence in each of the two reporters.

10. The method of claim 1, which further includes  
5 amplifying the target duplex nucleic acid in the structure prior to said adding.

11. The method of claim 1, which further includes  
10 amplifying the probe bound to the target by addition of polymerase, and all four deoxynucleotides, where one of the deoxynucleotides includes a reporter label.

12. A method of identifying the presence of a known  
15 viral nucleic target sequence contained in a fixed cellular or subcellular biological structure, comprising adding to the structure, a probe complex composed of RecA protein stably bound to a single-stranded, reporter-labeled probe which is complementary to the viral nucleic target sequence, under conditions in which the complex  
20 can contact the nucleic acid target, allowing the complex to bind to the target sequence under non-denaturing conditions, removing unbound complex from said structure, and  
25 examining the structure for the presence of the reporter-labeled probe bound to the nucleic acid.

13. The method of claim 12, where the known viral target is a sequence derived from hepatitis B virus.

30 14. The method of claim 12, where the fixed structures are incubated in 10 mM Tris-acetate buffer, pH 7.5, at 55-60°C before the addition of the RecA probe complex.

15. The method of claim 12, wherein the complex is stabilized by the presence of a cofactor selected from the group consisting of ATP $\gamma$ S, GTP $\gamma$ S, ATP, dATP and a combination of ATP $\gamma$ S and ADP.

5

16. The method of claim 12, wherein said probe is labeled with a ligand reporter, and said examining includes adding to the structure, specific ligand molecule, effective to stably bind to said ligand, and 10 having a detectable reporter group.

15

17. The method of claim 16, wherein said ligand reporter is digoxigenin or biotin and said ligand molecule is selected from the group consisting of an antibody, avidin and streptavidin.

20

18. The method of claim 12, wherein said examining includes detecting a fluorescent reporter bound to the reporter-labeled probe bound to the nucleic acid using either microscopy or a fluorescence activated cell sorter.

25

19. The method of claim 12, for localizing a selected target duplex nucleic acid sequence integrated into a host-cell genome, wherein said complex is added to the chromosomes of the cell, and said examining includes examining the chromosomes microscopically to determine the relative position of reporter-labeled probe in relation to chromosome ultrastructure.

30

20. A kit for the practice of the method of claim 12, comprising

a RecA-prot in coated DNA probe derived from the viral nucleic acid sequences.

35

21. The kit of claim 20, where the probe is derived from hepatitis B virus sequences.

5 22. The kit of claim 20, where the probe is reporter labeled.

23. The kit of claim 22, where the reporter is biotin or digoxigenin.

10 24. The kit of claim 20, where the kit further includes means of detecting the binding of the probe to the known viral nucleic acid sequences in a sample and said means of detection includes detecting a fluorescent reporter bound to the reporter-labeled probe bound to the nucleic acid using either microscopy or a fluorescence activated cell sorter.

20 25. A method of detecting a single copy nucleic acid sequence contained in a cellular or subcellular biological structure, comprising fixing the cellular or subcellular biological structure, adding to the structure, a probe complex composed of RecA protein stably bound to a single-stranded, reporter-labeled probe which is complementary to the single-copy nucleic acid target sequence, under conditions in which the complex can contact the nucleic acid target, allowing the complex to bind to the target sequence under non-denaturing conditions, removing unbound complex from said structure, and examining the structure for the presence of the reporter-labeled probe bound to the nucleic acid.

30 35 26. The method of claim 25, where said fixing is in solution or on a slide.

27. The method of claim 25, where said fixing includes incubation of the fixed structures in 10 mM Tris-acetate buffer, pH 7.5, at 55-60°C.

5 28. The method of claim 25, where said allowing the complex to bind to the target sequence under non-denaturing conditions is carried out for less than 2 hours.

10 29. The method of claim 25, where said adding includes the addition of accessory proteins.

15 30. The method of claim 29, where said accessory proteins are topoisomerase I or topoisomerase II.

31. The method of claim 1, where said fixed structures are in solution or on a slide.

20 32. The method of claim 1, where said fixed structures are incubated in 10 mM Tris-acetate buffer, pH 7.5, at 55-60°C before the addition of said RecA probe complex.

25 33. The method of claim 1, where said allowing the complex to bind to the target sequence under non-denaturing conditions is carried out for less than 2 hours.

30 34. The method of claim 1, where said adding includes the addition of accessory proteins.

35. The method of claim 34, where said accessory proteins are topoisomerase I or topoisomerase II.

35 36. The method of claim 12, where said fixed

structures are in solution or on a slide.

37. The method of claim 12, where said allowing the complex to bind to the target sequence under non-denaturing conditions is carried out for less than 2 hours.

38. The method of claim 12, where said adding includes the addition of accessory proteins.

10

39. The method of claim 38, where said accessory proteins are topoisomerase I or topoisomerase II.

40. The method of claim 25, wherein the complex is stabilized by the presence of a cofactor selected from the group consisting of ATP<sub>γ</sub>-S, GTP<sub>γ</sub>-S, ATP, dATP and a combination of ATP<sub>γ</sub>-S and ADP.

41. The method of claim 25, wherein said probe is labeled with a ligand reporter, and said examining includes adding to the structure, specific ligand molecule, effective to stably bind to said ligand, and having a detectable reporter group.

42. The method of claim 41, wherein said ligand reporter is digoxigenin or biotin and said ligand molecule is selected from the group consisting of an antibody, avidin and streptavidin.

43. The method of claim 25, wherein said examining includes detecting a fluorescent reporter bound to the reporter-labeled probe bound to the nucleic acid using either microscopy or a fluorescence activated cell sorter.

44. The method of claim 25, for localizing a selected target duplex nucleic acid sequence integrated into a host-cell genome, wherein said complex is added to the chromosomes of the cell, and said examining includes examining the chromosomes microscopically to determine the relative position of reporter-labeled probe in relation to chromosome ultrastructure.

45. A kit for the practice of the method of claim 25, comprising

a RecA-protein coated DNA probe derived from the single copy nucleic acid sequences.

46. The kit of claim 45, where the probe is derived from p53 tumor suppressor gene sequences.

47. The kit of claim 45, where the probe is reporter labeled.

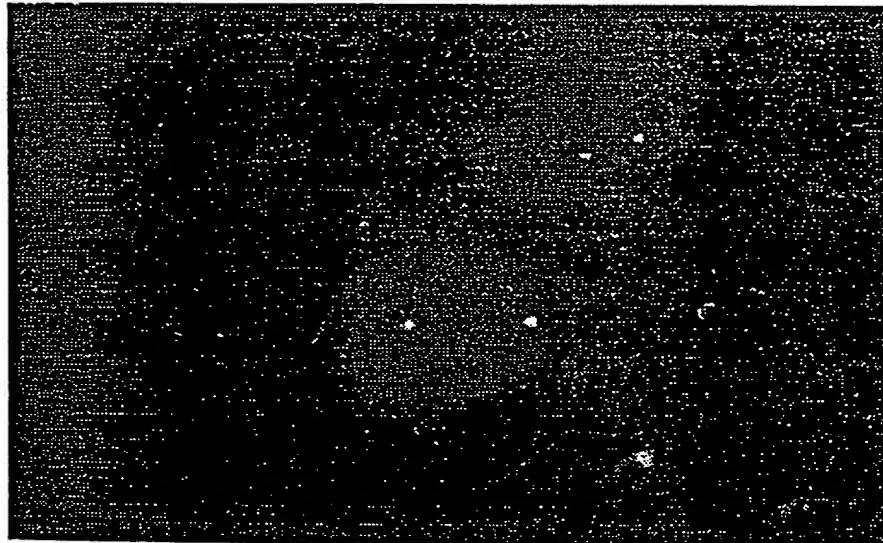
48. The kit of claim 47, where reporter is biotin or digoxigenin.

49. The kit of claim 45, where the kit further includes means of detecting the binding of the probe to the single copy nucleic acid sequences in a sample and said means of detection includes detecting a fluorescent reporter bound to the reporter-labeled probe bound to the nucleic acid using either microscopy or a fluorescence activated cell sorter.

1/3



**Fig. 1A**



**Fig. 1B**

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2/3

Fig. 2A

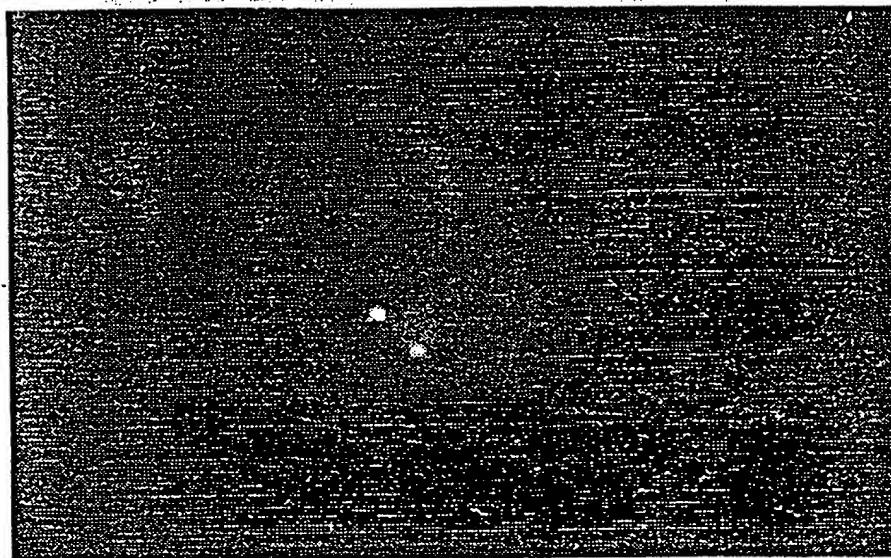
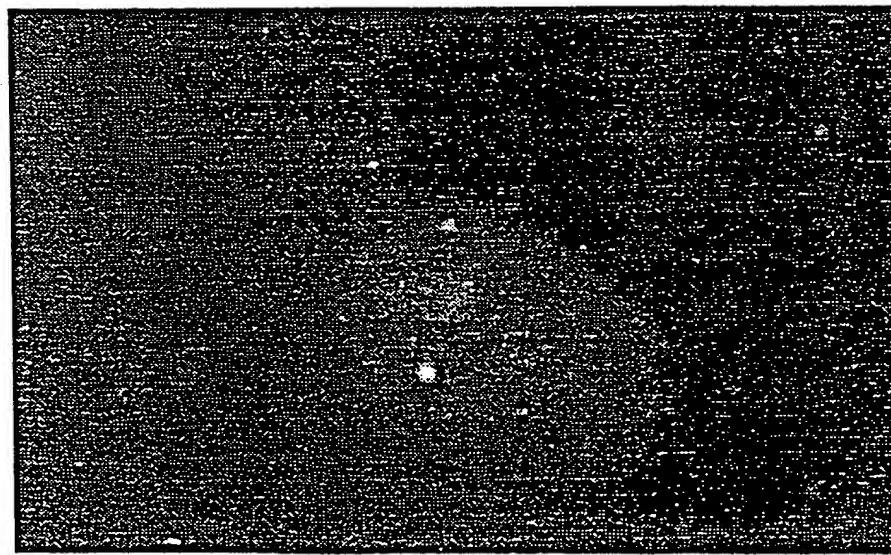


Fig. 2B



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3/3

Fig. 3A

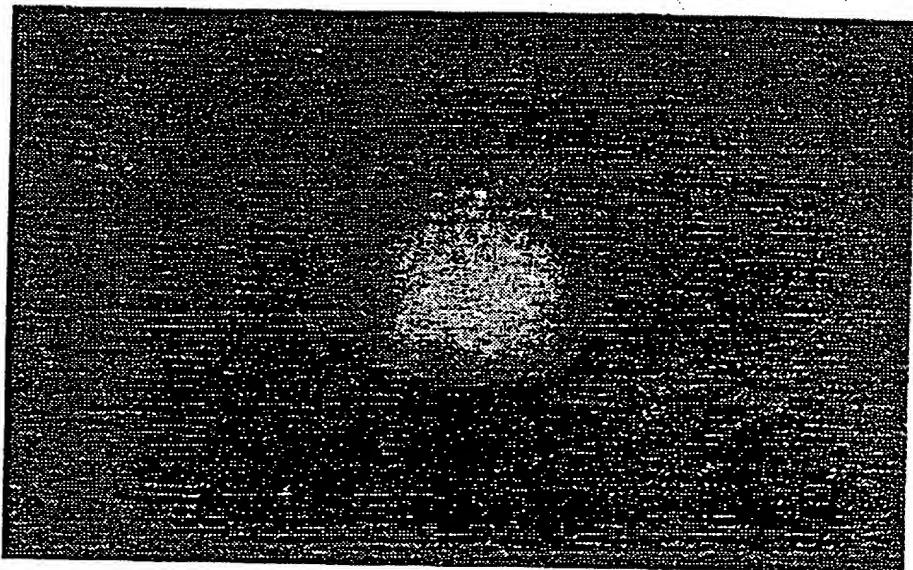
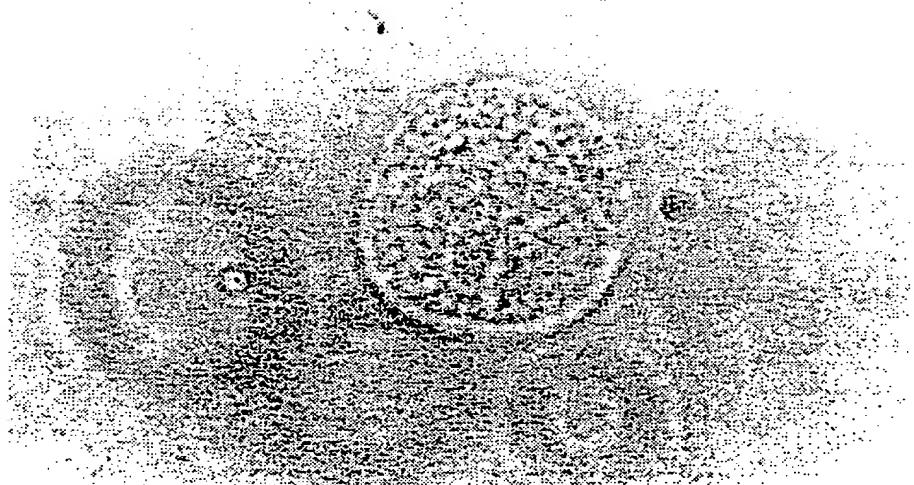


Fig. 3B



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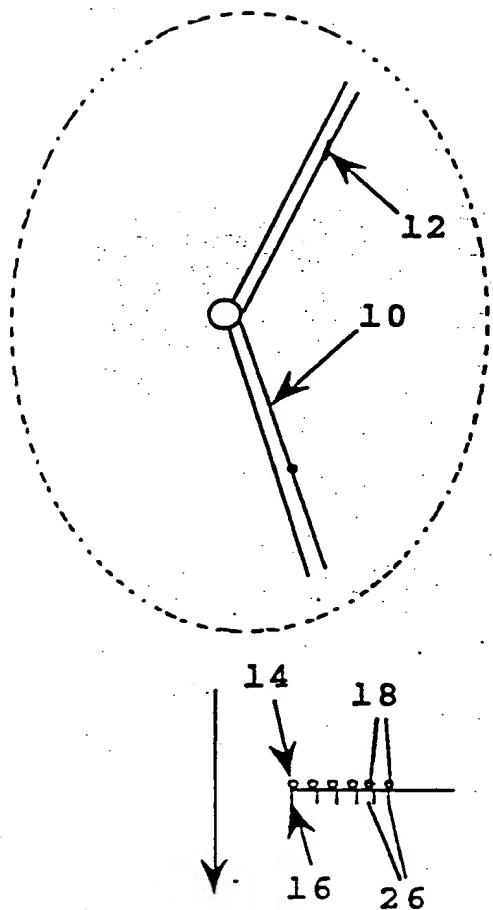


Fig. 4A

4/3

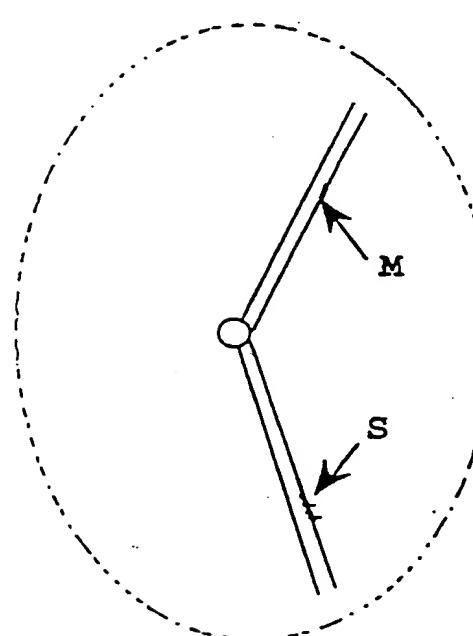


Fig. 4B

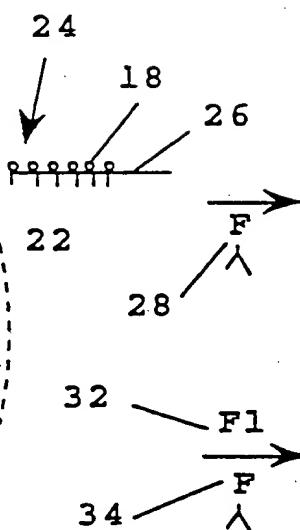


Fig. 4C

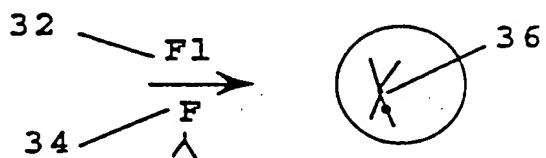


Fig. 4D

Fig. 5A

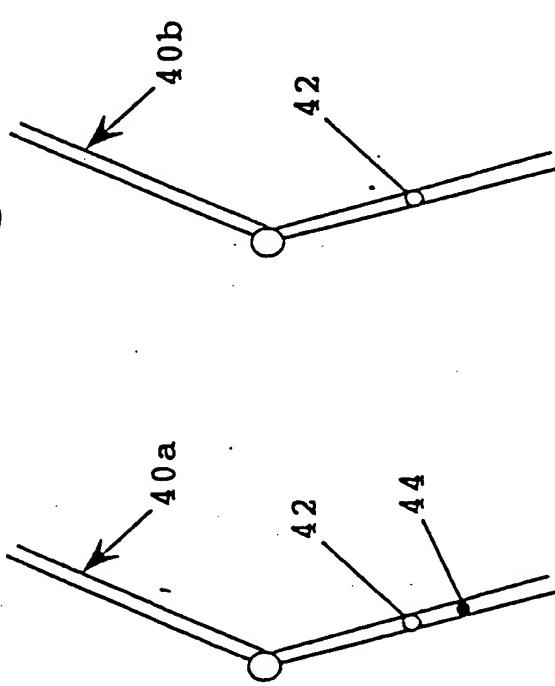


Fig. 5B

Fig. 6A

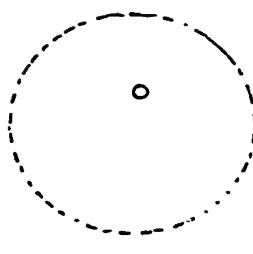
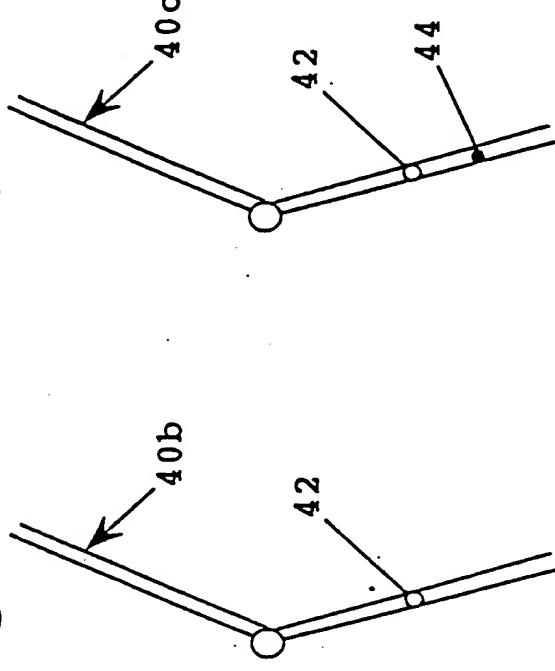


Fig. 6B

Fig. 7A

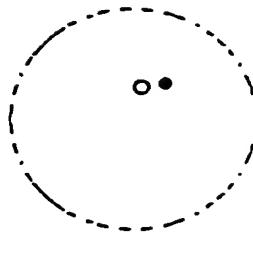
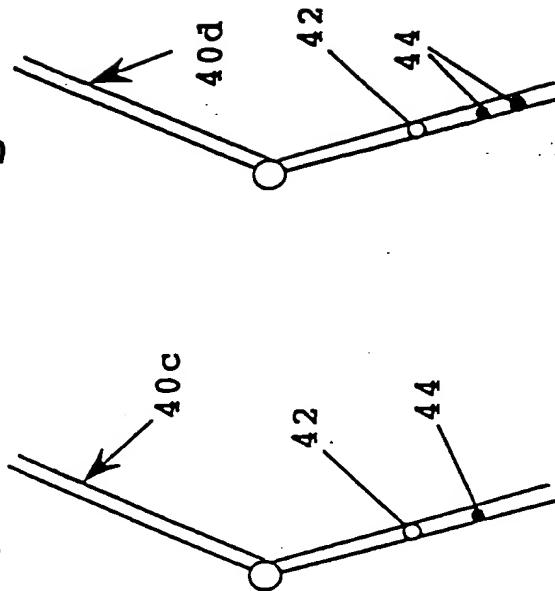


Fig. 7B

Fig. 8A

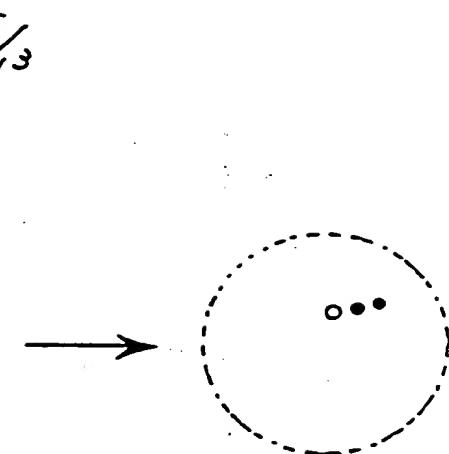
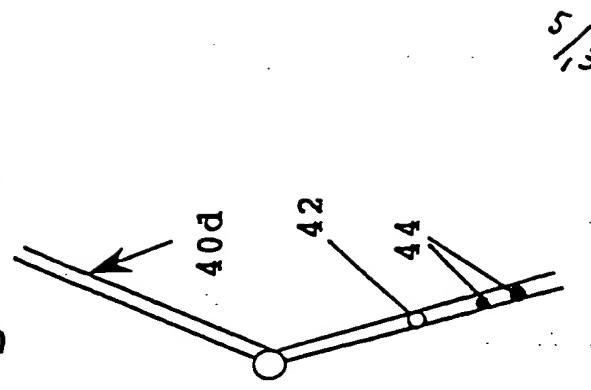


Fig. 8B

6/3

Fig. 9A

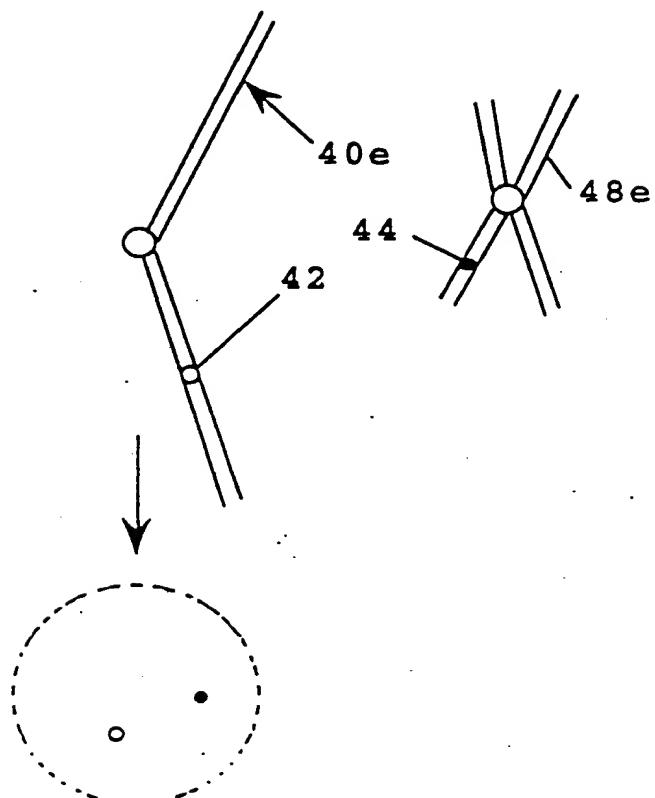


Fig. 10A

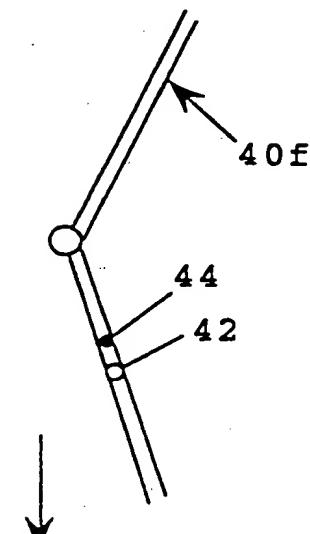


Fig. 9B

Fig. 10B

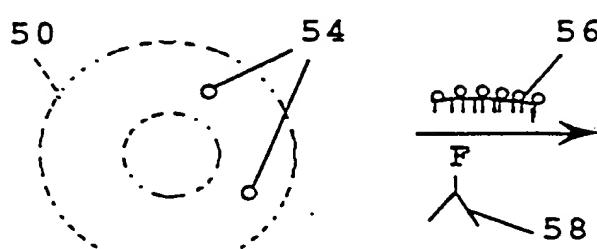


Fig. 11A

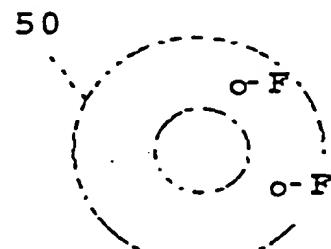


Fig. 11B

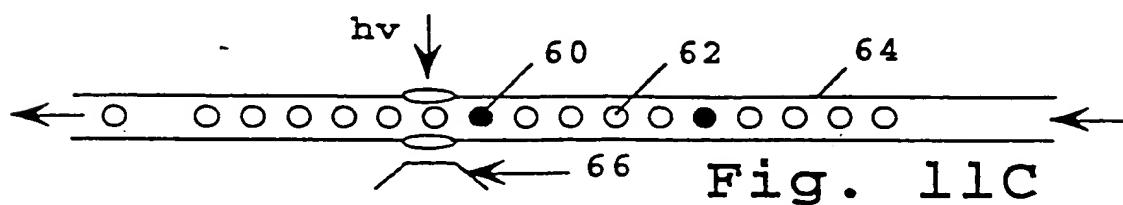


Fig. 11C

7/13

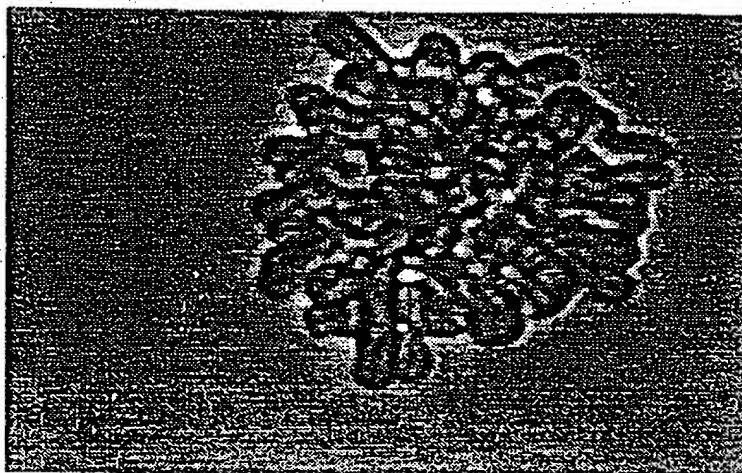


Fig. 12

**SUBSTITUTE SHEET**

8  
1/2



Fig. 13A

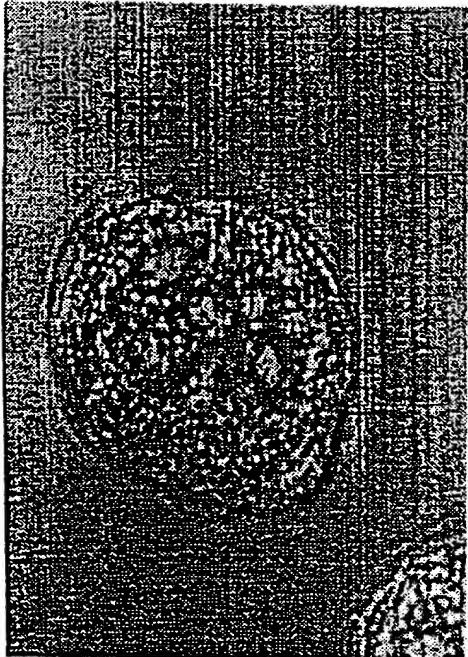


Fig. 13B

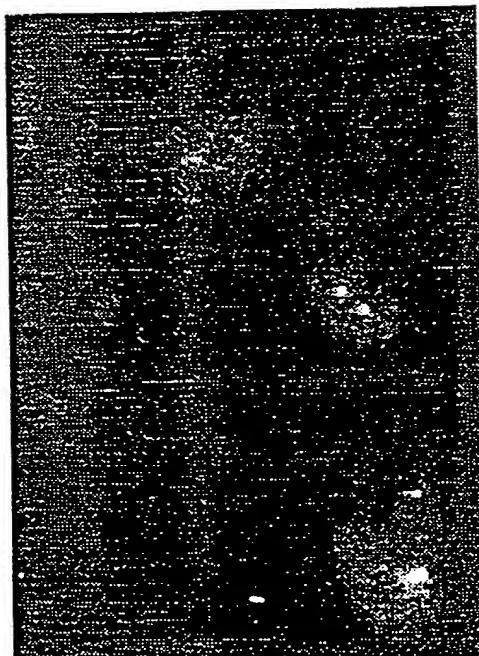


Fig. 13C

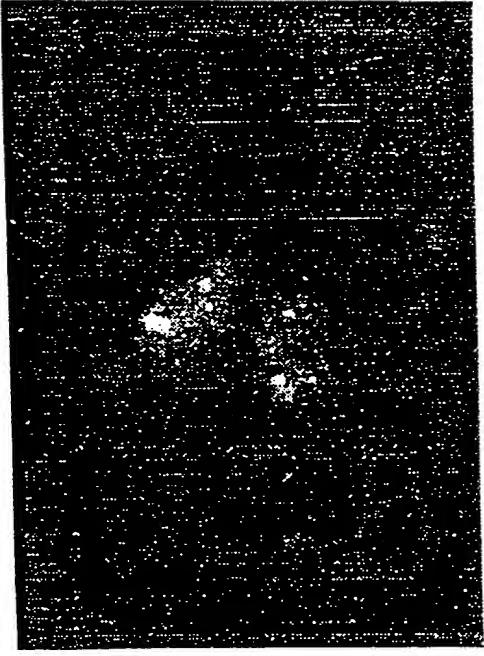


Fig. 13D

9/13

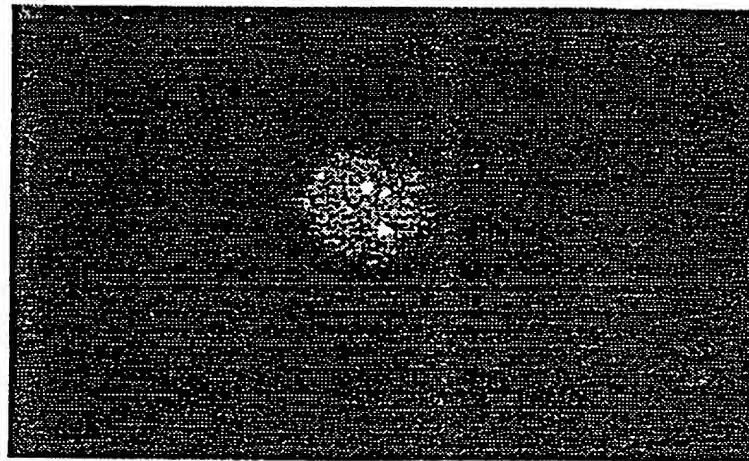


Fig. 13E

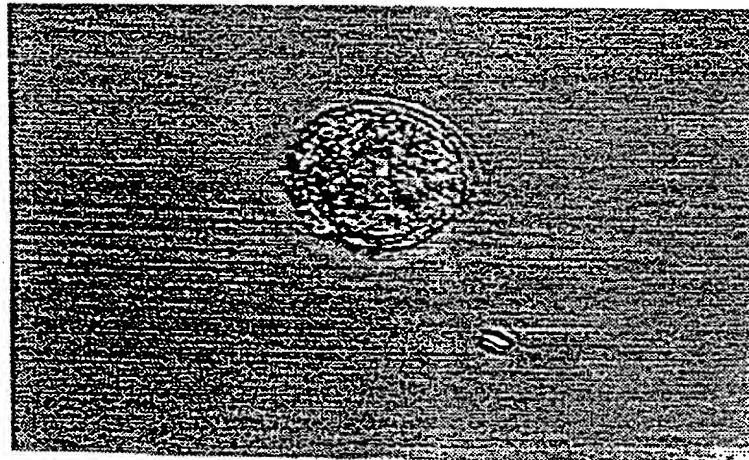


Fig. 13F

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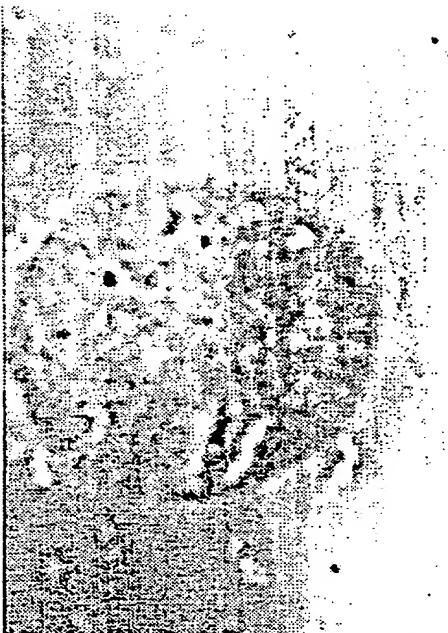


Fig. 14A

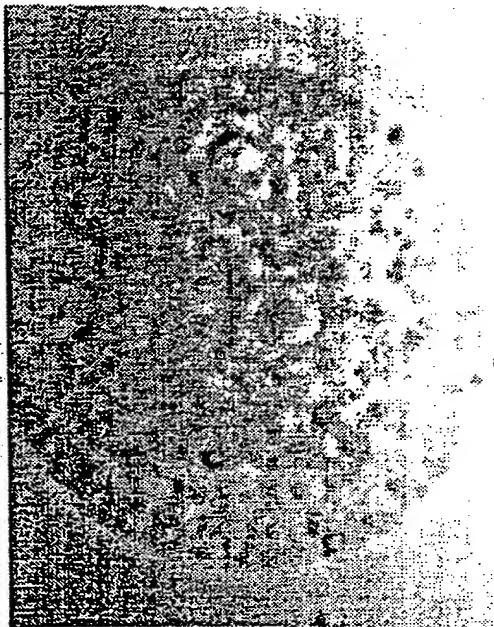


Fig. 14B

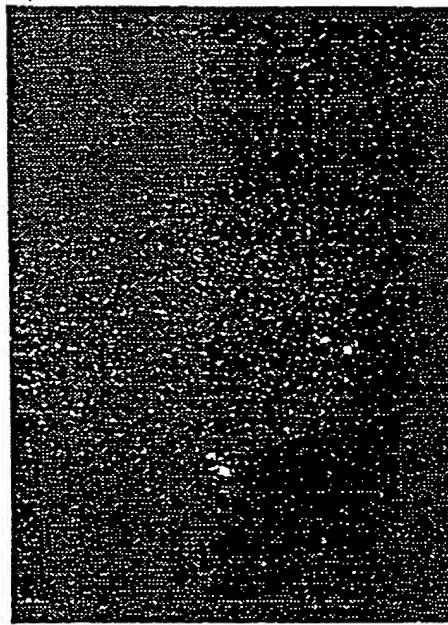


Fig. 14C

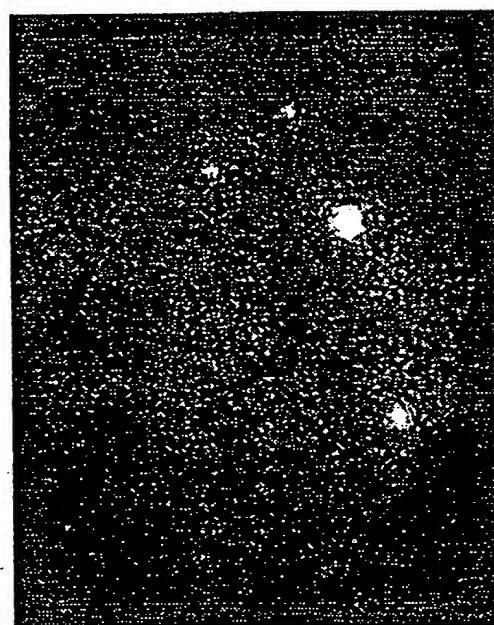


Fig. 14D

11  
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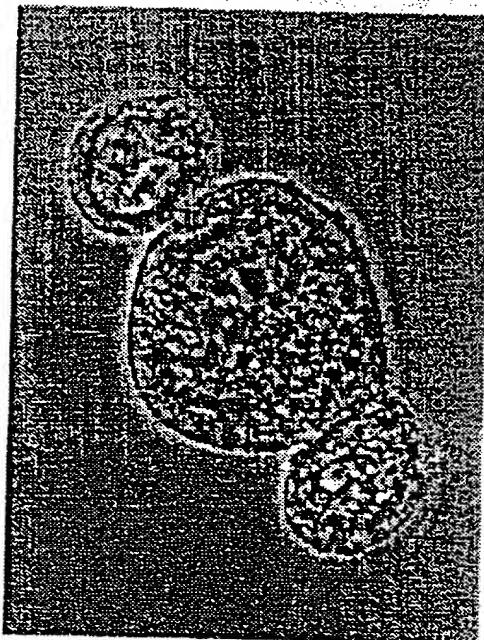


Fig. 15A

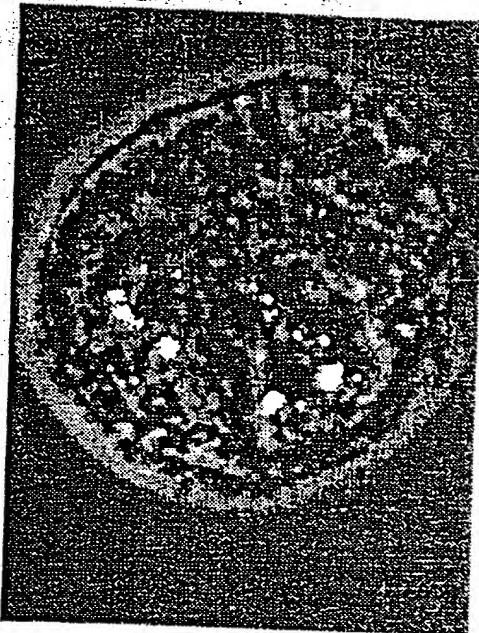


Fig. 15B

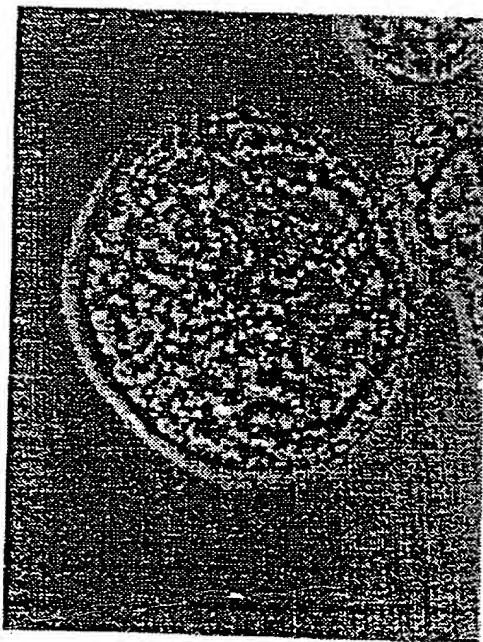
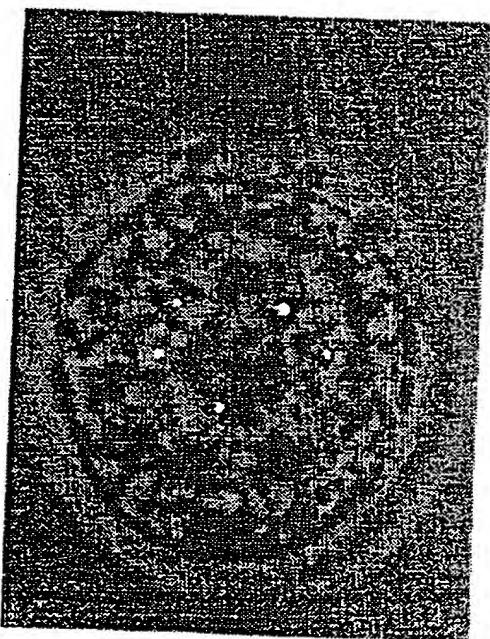


Fig. 15C

Fig. 15D



SUBSTITUTE SHEET

12/13

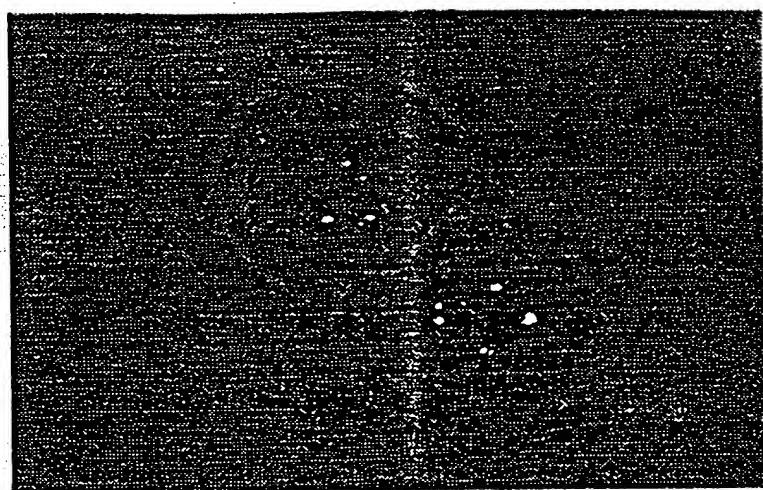


Fig. 15E

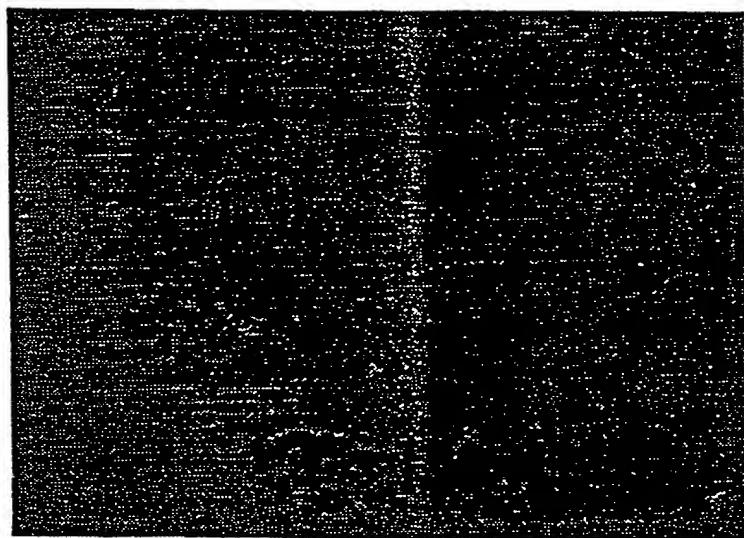


Fig. 16A

13/13



Fig. 16B

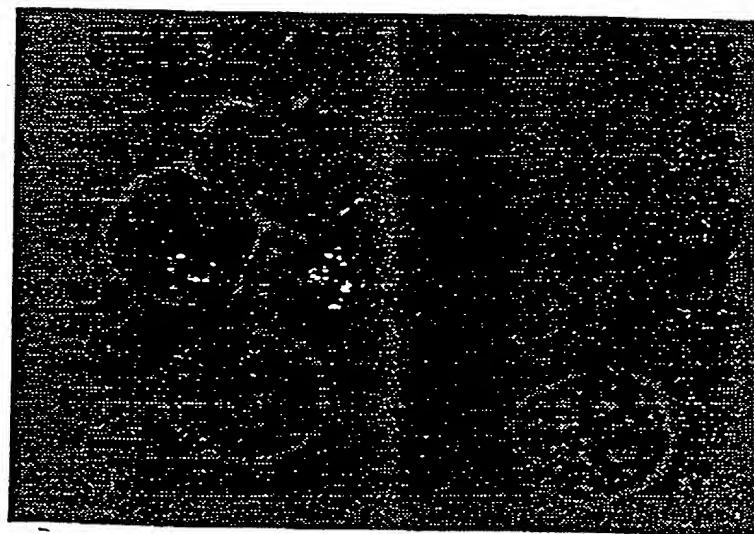


Fig. 16C

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## INTERNATIONAL SEARCH REPORT

International Application No PCT/JP 92/01128

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 Q 1/68

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols
IPC5	C 12 Q
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched <sup>8</sup>	

III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	WO, A1, 8701730 (YALE UNIVERSITY) 26 March 1987, see especially the abstract and the claims --	1-49
P, A	WO, A1, 9117267 (SRI INTERNATIONAL) 14 November 1991, see the whole document --	1-49
A	EP, A2, 0322311 (APPLIED BIOSYSTEMS, INC.) 28 June 1989, see the whole document --	1-49

<sup>10</sup> Special categories of cited documents:<sup>11</sup> "A" document defining the general state of the art which is not considered to be of particular relevance<sup>12</sup> "E" earlier document but published on or after the international filing date<sup>13</sup> "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<sup>14</sup> "O" document referring to an oral disclosure, use, exhibition or other means<sup>15</sup> "P" document published prior to the international filing date but later than the priority date claimed<sup>16</sup> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<sup>17</sup> "X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step<sup>18</sup> "Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<sup>19</sup> "&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

7th January 1993

Date of Mailing of this International Search Report

26.01.93

International Searching Authority

Signature of Authorized Officer

EUROPEAN PATENT OFFICE

Mikael G:son Bergstrand

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	WO, A1, 8505685 (NATIONAL BIOMEDICAL RESEARCH FOUNDATION) 19 December 1985, see the whole document -----	1-49

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.PCT/JP 92/01128

SA 65632

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The members are as contained in the European Patent Office EDP file on 02/12/92  
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 8701730	26/03/87	AU-D- EP-A- US-A-	6403886 0236491 4888274	07/04/87 16/09/87 19/12/89
WO-A1- 9117267	14/11/91	CA-A- EP-A-	2056983 0481065	08/11/91 22/04/92
EP-A2- 0322311	28/06/89	JP-A-	1289499	21/11/89
WO-A1- 8505685	19/12/85	EP-A- JP-T-	0183822 61502282	11/06/86 09/10/86

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